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(54) Title: REGULATION OF HUMAN CATION TRANSPORT ATPASE-LIKE PROTEIN

(57) Abstract: Reagents that regulate human cation transport ATPase-like protein and reagents which bind to human cation transport ATPase-like gene products can play a role in preventing, ameliorating, or correcting dysfunctions or diseases including, but not limited to, CNS disorders, cardiovascular disorders, cancer, genitourinary disorders, and hematological disorders.

REGULATION OF HUMAN CATION TRANSPORT ATPase-LIKE PROTEIN

This application incorporates by reference co-pending US provisional applications Serial No. 60/357,840 filed February 21, 2002; Serial No. 60/378,654 filed May 9, 5 Serial No. 60/406,658 filed August 29, 2002.

FIELD OF THE INVENTION

The invention relates to the regulation of human cation transport ATPase-like 10 protein.

BACKGROUND OF THE INVENTION

ATPases are involved in a variety of metabolic processes (1-4). There is a need in the 15 art to identify related proteins, which can be regulated to provide therapeutic effects.

BRIEF SUMMARY OF THE INVENTION

It is an object of the invention to provide reagents and methods of regulating a 20 human cation transport ATPase-like protein. This and other objects of the invention are provided by one or more of the embodiments described below.

One embodiment of the invention is a cation transport ATPase-like protein polypeptide comprising an amino acid sequence selected from the group consisting of:

25 amino acid sequences which are at least about 95% identical to the amino acid sequence shown in SEQ ID NO: 2; and
the amino acid sequence shown in SEQ ID NO: 2.

30 Yet another embodiment of the invention is a method of screening for agents which decrease the activity of cation transport ATPase-like protein polypeptide. A test

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compound is contacted with a cation transport ATPase-like protein polypeptide comprising an amino acid sequence selected from the group consisting of:

5. amino acid sequences which are at least about 95% identical to the amino acid sequence shown in SEQ ID NO: 2; and
the amino acid sequence shown in SEQ ID NO: 2.

10 Binding between the test compound and the cation transport ATPase-like protein polypeptide is detected. A test compound which binds to the cation transport ATPase-like protein polypeptide is thereby identified as a potential agent for decreasing the activity of cation transport ATPase-like protein polypeptide.

15 Another embodiment of the invention is a method of screening for agents which decrease the activity of cation transport ATPase-like protein polypeptide. A test compound is contacted with a polynucleotide encoding a cation transport ATPase-like protein polypeptide, wherein the polynucleotide comprises a nucleotide sequence selected from the group consisting of:

20 nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO: 1;
the nucleotide sequence shown in SEQ ID NO: 1;

25 nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO: 3; and
the nucleotide sequence shown in SEQ ID NO: 3.

30 Binding of the test compound to the polynucleotide is detected. A test compound which binds to the polynucleotide is identified as a potential agent for decreasing the activity of cation transport ATPase-like protein polypeptide. The agent can work by decreasing the amount of the cation transport ATPase-like protein through interacting with the cation transport ATPase-like protein polypeptide mRNA.

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5 Another embodiment of the invention is a method of screening for agents which regulate the activity of cation transport ATPase-like protein polypeptide. A test compound is contacted with a cation transport ATPase-like protein polypeptide comprising an amino acid sequence selected from the group consisting of:

10 amino acid sequences which are at least about 95% identical to the amino acid sequence shown in SEQ ID NO: 2; and
the amino acid sequence shown in SEQ ID NO: 2.

15 A cation transport ATPase-like protein activity of the polypeptide is detected. A test compound which increases cation transport ATPase-like protein activity of the polypeptide relative to cation transport ATPase-like protein activity in the absence of the test compound is thereby identified as a potential agent for increasing the activity of cation transport ATPase-like protein polypeptide. A test compound which decreases cation transport ATPase-like protein activity of the polypeptide relative to cation transport ATPase-like protein activity in the absence of the test compound is thereby identified as a potential agent for decreasing the activity of cation transport ATPase-like protein polypeptide.

20 Even another embodiment of the invention is a method of screening for agents which decrease the activity of cation transport ATPase-like protein polypeptide. A test compound is contacted with a cation transport ATPase-like protein product of a polynucleotide which comprises a nucleotide sequence selected from the group 25 consisting of:

nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO: 1;
the nucleotide sequence shown in SEQ ID NO: 1;

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nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO: 3; and
the nucleotide sequence shown in SEQ ID NO: 3.

5 Binding of the test compound to the cation transport ATPase-like protein product is detected. A test compound which binds to the cation transport ATPase-like protein product is thereby identified as a potential agent for decreasing the activity of cation transport ATPase-like protein polypeptide.

10 Still another embodiment of the invention is a method of reducing the activity of cation transport ATPase-like protein polypeptide. A cell is contacted with a reagent which specifically binds to a polynucleotide encoding a cation transport ATPase-like protein polypeptide or the product encoded by the polynucleotide, wherein the polynucleotide comprises a nucleotide sequence selected from the group consisting of:

15 nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO: 1;
the nucleotide sequence shown in SEQ ID NO: 1;

20 nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO: 3; and
the nucleotide sequence shown in SEQ ID NO: 3.

Cation transport ATPase-like protein activity in the cell is thereby decreased.

25 The invention thus provides a human cation transport ATPase-like protein that can be used to identify test compounds that may act, for example, as activators or inhibitors at the enzyme's active site. Human cation transport ATPase-like protein and fragments thereof also are useful in raising specific antibodies that can block the enzyme and effectively reduce its activity.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to an isolated polynucleotide from the group consisting of:

5 a) a polynucleotide encoding a cation transport ATPase-like protein polypeptide comprising an amino acid sequence selected from the group consisting of:

amino acid sequences which are at least about 95% identical to the amino acid sequence shown in SEQ ID NO: 2; and

10 the amino acid sequence shown in SEQ ID NO: 2.

b) a polynucleotide comprising the sequence of SEQ ID NO: 1 or 3;

15 c) a polynucleotide which hybridizes under stringent conditions to a polynucleotide specified in (a) and (b) and encodes a cation transport ATPase-like protein polypeptide;

20 d) a polynucleotide the sequence of which deviates from the polynucleotide sequences specified in (a) to (c) due to the degeneration of the genetic code and encodes a cation transport ATPase-like protein polypeptide; and

25 e) a polynucleotide which represents a fragment, derivative or allelic variation of a polynucleotide sequence specified in (a) to (d) and encodes a cation transport ATPase-like protein polypeptide.

Furthermore, it has been discovered by the present applicant that a novel cation transport ATPase-like protein, particularly a human cation transport ATPase-like protein, can be used in therapeutic methods to treat CNS disorders, cardiovascular disorders, cancer, genitourinary disorders and hematological disorders. Human cation transport ATPase-like protein comprises the amino acid sequence shown in SEQ ID NO: 2. A DNA harboring the coding sequence (ORF) for human cation transport

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ATPase-like protein is shown in SEQ ID NO: 1. The ORF is shown in SEQ ID NO: 3. Its gene maps to chromosome 13q12.2.

5 Human cation transport ATPase-like protein also can be used to screen for human cation transport ATPase-like protein activators and inhibitors.

Polypeptides

10 Human cation transport ATPase-like polypeptides according to the invention comprise at least 6, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, or 1148 contiguous amino acids selected from the amino acid sequence shown in SEQ ID NO: 2 or a biologically active variant thereof, as defined below. A cation transport ATPase-like polypeptide of the invention therefore can be a portion of a cation transport ATPase-like protein, 15 a full-length cation transport ATPase-like protein, or a fusion protein comprising all or a portion of a cation transport ATPase-like protein.

Biologically active variants

20 Human cation transport ATPase-like polypeptide variants which are biologically active, e.g., retain enzymatic activity also are human cation transport ATPase-like polypeptides. Preferably, naturally or non-naturally occurring human cation transport ATPase-like polypeptide variants have amino acid sequences which are at least about 95, 96, 97, 98, or 99% identical to the amino acid sequence shown in 25 SEQ ID NO: 2 or a fragment thereof. Percent identity between a putative human cation transport ATPase-like polypeptide variant and an amino acid sequence of SEQ ID NO: 2 is determined by conventional methods. See, for example, Altschul *et al.*, *Bull. Math. Bio.* 48:603 (1986), and Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the 30 alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM62" scoring matrix of Henikoff & Henikoff, 1992.

Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson & Lipman is a suitable protein alignment method for examining 5 the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative variant. The FASTA algorithm is described by Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444(1988), and by Pearson, *Meth. Enzymol.* 183:63 (1990). Briefly, FASTA first characterizes sequence 10 similarity by identifying regions shared by the query sequence (e.g., SEQ ID NO: 2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest 15 density of identities are then rescored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring 20 regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch- Sellers algorithm (Needleman & Wunsch, *J. Mol. Biol.* 48:444 (1970); Sellers, *SIAM J. Appl. Math.* 26:787 (1974)), which allows for amino acid insertions and deletions. Preferred parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. 25 These parameters can be introduced into a FASTA program by modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol.* 183:63 (1990).

FASTA can also be used to determine the sequence identity of nucleic acid 30 molecules using a ratio as disclosed above. For nucleotide sequence comparisons,

the *ktup* value can range between one to six, preferably from three to six, most preferably three, with other parameters set as default.

5 Variations in percent identity can be due, for example, to amino acid substitutions, insertions, or deletions. Amino acid substitutions are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

10 Amino acid insertions or deletions are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological or immunological activity of a human cation transport ATPase-like 15 polypeptide can be found using computer programs well known in the art, such as DNASTAR software.

20 The invention additionally, encompasses cation transport ATPase-like polypeptides that are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications can be carried out by known 25 techniques including, but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc.

30 Additional post-translational modifications encompassed by the invention include, for example, *e.g.*, N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of prokaryotic

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host cell expression. The cation transport ATPase-like polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

5 The invention also provides chemically modified derivatives of cation transport ATPase-like polypeptides that may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (see U.S. Patent No. 4,179,337). The chemical moieties for derivitization can be selected from water soluble polymers such as polyethylene glycol, ethylene 10 glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol, and the like. The polypeptides can be modified at random or predetermined positions within the molecule and can include one, two, three, or more attached chemical moieties.

15 Whether an amino acid change or a polypeptide modification results in a biologically active cation transport ATPase-like polypeptide can readily be determined by assaying for enzymatic activity, as described for example, in J Biochem Mol Toxicol 1999;13(2):93-106.

20 *Fusion proteins*

25 Fusion proteins are useful for generating antibodies against cation transport ATPase-like polypeptide amino acid sequences and for use in various assay systems. For example, fusion proteins can be used to identify proteins that interact with portions of a human cation transport ATPase-like polypeptide. Protein affinity chromatography or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage display systems, can be used for this purpose. Such methods are well known in the art and also can be used as drug screens.

30 A human cation transport ATPase-like polypeptide fusion protein comprises two polypeptide segments fused together by means of a peptide bond. The first

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polypeptide segment comprises at least 6, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, or 1148 contiguous amino acids of SEQ ID NO: 2 or of a biologically active variant, such as those described above. The first polypeptide segment also can comprise full-length 5 cation transport ATPase-like protein.

The second polypeptide segment can be a full-length protein or a protein fragment. Proteins commonly used in fusion protein construction include β -galactosidase, β -glucuronidase, green fluorescent protein (GFP), autofluorescent proteins, including 10 blue fluorescent protein (BFP), glutathione-S-transferase (GST), luciferase, horseradish peroxidase (HRP), and chloramphenicol acetyltransferase (CAT). Additionally, epitope tags are used in fusion protein constructions, including histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Other fusion constructions can include maltose binding 15 protein (MBP), S-tag, Lex a DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. A fusion protein also can be engineered to contain a cleavage site located between the cation transport ATPase-like polypeptide-encoding sequence and the heterologous protein sequence, so that the cation transport ATPase-like polypeptide can be cleaved 20 and purified away from the heterologous moiety.

A fusion protein can be synthesized chemically, as is known in the art. Preferably, a fusion protein is produced by covalently linking two polypeptide segments or by standard procedures in the art of molecular biology. Recombinant DNA methods can 25 be used to prepare fusion proteins, for example, by making a DNA construct which comprises coding sequences selected from SEQ ID NO: 1 or 3 in proper reading frame with nucleotides encoding the second polypeptide segment and expressing the DNA construct in a host cell, as is known in the art. Many kits for constructing fusion proteins are available from companies such as Promega Corporation (Madison, WI), Stratagene (La Jolla, CA), CLONTECH (Mountain View, CA), 30 Santa Cruz Biotechnology (Santa Cruz, CA); MBL International Corporation (MIC;

Watertown, MA), and Quantum Biotechnologies (Montreal, Canada; 1-888-DNA-KITS).

Identification of species homologs

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Species homologs of human cation transport ATPase-like polypeptide can be obtained using cation transport ATPase-like polypeptide polynucleotides (described below) to make suitable probes or primers for screening cDNA expression libraries from other species, such as mice, monkeys, or yeast, identifying cDNAs which encode homologs of cation transport ATPase-like polypeptide, and expressing the cDNAs as is known in the art.

10

Polynucleotides

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A human cation transport ATPase-like polynucleotide can be single- or double-stranded and comprises a coding sequence or the complement of a coding sequence for a cation transport ATPase-like polypeptide. A coding sequence for human cation transport ATPase-like protein is shown in SEQ ID NO: 3.

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Degenerate nucleotide sequences encoding human cation transport ATPase-like polypeptides, as well as homologous nucleotide sequences which are at least about 50, 55, 60, 65, 70, preferably about 75, 90, 96, 98, or 99% identical to the nucleotide sequence shown in SEQ ID NO: 1 or 3 or their complements also are cation transport ATPase-like polynucleotides. Percent sequence identity between the sequences of two polynucleotides is determined using computer programs such as ALIGN which employ the FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2. Complementary DNA (cDNA) molecules, species homologs, and variants of cation transport ATPase-like polynucleotides that encode biologically active cation transport ATPase-like polypeptides also are cation transport ATPase-like polynucleotides. Polynucleotide fragments comprising at least 8, 9, 10, 11, 12, 15, 20, or 25 contiguous nucleotides of SEQ ID NO: 1 or 3 or their

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complements also are cation transport ATPase-like polynucleotides. These fragments can be used, for example, as hybridization probes or as antisense oligonucleotides.

5 *Identification of polynucleotide variants and homologs*

Variants and homologs of the cation transport ATPase-like polynucleotides described above also are cation transport ATPase-like polynucleotides. Typically, homologous cation transport ATPase-like polynucleotide sequences can be identified by 10 hybridization of candidate polynucleotides to known cation transport ATPase-like polynucleotides under stringent conditions, as is known in the art. For example, using the following wash conditions—2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 30 minutes each; then 2X SSC, 0.1% SDS, 50°C once, 30 minutes; then 2X SSC, room temperature twice, 10 minutes 15 each—homologous sequences can be identified which contain at most about 25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

Species homologs of the cation transport ATPase-like polynucleotides disclosed 20 herein also can be identified by making suitable probes or primers and screening cDNA expression libraries from other species, such as mice, monkeys, or yeast. Human variants of cation transport ATPase-like polynucleotides can be identified, for example, by screening human cDNA expression libraries. It is well known that 25 the T_m of a double-stranded DNA decreases by 1-1.5°C with every 1% decrease in homology (Bonner *et al.*, *J. Mol. Biol.* 81, 123 (1973)). Variants of human cation transport ATPase-like polynucleotides or cation transport ATPase-like polynucleotides of other species can therefore be identified by hybridizing a putative homologous cation transport ATPase-like polynucleotide with a polynucleotide 30 having a nucleotide sequence of SEQ ID NO: 1 or 3 or the complement thereof to form a test hybrid. The melting temperature of the test hybrid is compared with the melting temperature of a hybrid comprising polynucleotides having perfectly

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complementary nucleotide sequences, and the number or percent of basepair mismatches within the test hybrid is calculated.

5 Nucleotide sequences which hybridize to cation transport ATPase-like polynucleotides or their complements following stringent hybridization and/or wash conditions also are cation transport ATPase-like polynucleotides. Stringent wash conditions are well known and understood in the art and are disclosed, for example, in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed., 1989, at pages 9.50-9.51.

10 Typically, for stringent hybridization conditions a combination of temperature and salt concentration should be chosen that is approximately 12-20°C below the calculated T_m of the hybrid under study. The T_m of a hybrid between a cation transport ATPase-like polynucleotide having a nucleotide sequence shown in SEQ 15 ID NO: 1 or 3 or the complement thereof and a polynucleotide sequence which is at least about 50, preferably about 75, 90, 96, or 98% identical to one of those nucleotide sequences can be calculated, for example, using the equation of Bolton and McCarthy, *Proc. Natl. Acad. Sci. U.S.A.* 48, 1390 (1962):

20 $T_m = 81.5^\circ\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%G + C) - 0.63(\%\text{formamide}) - 600/l$,
where l = the length of the hybrid in basepairs.

25 Stringent wash conditions include, for example, 4X SSC at 65°C, or 50% formamide, 4X SSC at 42°C, or 0.5X SSC, 0.1% SDS at 65°C. Highly stringent wash conditions include, for example, 0.2X SSC at 65°C.

Preparation of polynucleotides

30 A human cation transport ATPase-like polynucleotide can be isolated free of other cellular components such as membrane components, proteins, and lipids. Polynucleotides can be made by a cell and isolated using standard nucleic acid

purification techniques, or synthesized using an amplification technique, such as the polymerase chain reaction (PCR), or by using an automatic synthesizer. Methods for isolating polynucleotides are routine and are known in the art. Any such technique for obtaining a polynucleotide can be used to obtain isolated cation transport 5 ATPase-like polynucleotides. For example, restriction enzymes and probes can be used to isolate polynucleotide fragments, which comprise cation transport ATPase-like protein nucleotide sequences. Isolated polynucleotides are in preparations that are free or at least 70, 80, or 90% free of other molecules.

10 Human cation transport ATPase-like cDNA molecules can be made with standard molecular biology techniques, using cation transport ATPase-like mRNA as a template. Human cation transport ATPase-like cDNA molecules can thereafter be replicated using molecular biology techniques known in the art and disclosed in manuals such as Sambrook *et al.* (1989). An amplification technique, such as PCR, 15 can be used to obtain additional copies of polynucleotides of the invention, using either human genomic DNA or cDNA as a template.

20 Alternatively, synthetic chemistry techniques can be used to synthesize cation transport ATPase-like polynucleotides. The degeneracy of the genetic code allows alternate nucleotide sequences to be synthesized which will encode a human cation transport ATPase-like polypeptide having, for example, an amino acid sequence shown in SEQ ID NO: 2 or a biologically active variant thereof.

Extending polynucleotides

25 Various PCR-based methods can be used to extend the nucleic acid sequences disclosed herein to detect upstream sequences such as promoters and regulatory elements. For example, restriction-site PCR uses universal primers to retrieve unknown sequence adjacent to a known locus. Sarkar, *PCR Methods Applic.* 2, 30 318-322, 1993; Triglia *et al.*, *Nucleic Acids Res.* 16, 8186, 1988; Lagerstrom *et al.*, *PCR Methods Applic.* 1, 111-119, 1991; Parker *et al.*, *Nucleic Acids Res.* 19,

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3055-3060, 1991). Additionally, PCR, nested primers, and PROMOTERFINDER libraries (CLONTECH, Palo Alto, Calif.) can be used to walk genomic DNA (CLONTECH, Palo Alto, Calif.). See WO 01/98340

5 *Obtaining Polynucleotides*

Human cation transport ATPase-like polypeptides can be obtained, for example, by purification from human cells, by expression of cation transport ATPase-like polynucleotides, or by direct chemical synthesis.

10

Protein purification

Human cation transport ATPase-like polypeptides can be purified from any human cell which expresses the receptor, including host cells which have been transfected 15 with cation transport ATPase-like polynucleotides. A purified cation transport ATPase-like polypeptide is separated from other compounds that normally associate with the cation transport ATPase-like polypeptide in the cell, such as certain proteins, carbohydrates, or lipids, using methods well-known in the art. Such methods include, but are not limited to, size exclusion chromatography, ammonium sulfate 20 fractionation, ion exchange chromatography, affinity chromatography, and preparative gel electrophoresis.

A preparation of purified cation transport ATPase-like polypeptides is at least 80% pure; preferably, the preparations are 90%, 95%, or 99% pure. Purity of the 25 preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis.

Expression of polynucleotides

30 To express a human cation transport ATPase-like polynucleotide, the polynucleotide can be inserted into an expression vector which contains the necessary elements for

the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled in the art can be used to construct expression vectors containing sequences encoding cation transport ATPase-like polypeptides and appropriate transcriptional and translational control elements. These methods 5 include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook *et al.* (1989) and in Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, N.Y., 1989.

10 A variety of expression vector/host systems can be utilized to contain and express sequences encoding a human cation transport ATPase-like polypeptide. These include, but are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus 15 expression vectors (e.g., baculovirus), plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids), or animal cell systems. See WO 01/98340.

20 *Host cells*

A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed cation transport ATPase-like polypeptide in the desired fashion. Such modifications of the polypeptide include, 25 but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different host cells that have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and 30 WI38) are available from the American Type Culture Collection (ATCC; 10801

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University Boulevard, Manassas, VA 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein. See WO 01/98340.

5 Alternatively, host cells which contain a human cation transport ATPase-like polynucleotide and which express a human cation transport ATPase-like polypeptide can be identified by a variety of procedures known to those of skill in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). Hampton *et al.*, SEROLOGICAL METHODS: A LABORATORY MANUAL, APS Press, St. Paul, Minn., 10 Maddox *et al.*, *J. Exp. Med.* 158, 1211-1216, 1983). See WO 01/98340.

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to 15 polynucleotides encoding cation transport ATPase-like polypeptides include oligo-labeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, sequences encoding a human cation transport ATPase-like polypeptide can be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and can be used to 20 synthesize RNA probes *in vitro* by addition of labeled nucleotides and an appropriate RNA polymerase such as T7, T3, or SP6. These procedures can be conducted using a variety of commercially available kits (Amersham Pharmacia Biotech, Promega, and US Biochemical). Suitable reporter molecules or labels which can be used for ease of detection include radionuclides, enzymes, and fluorescent, chemiluminescent, 25 or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Expression and purification of polypeptides

30 Host cells transformed with nucleotide sequences encoding a human cation transport ATPase-like polypeptide can be cultured under conditions suitable for the expression

and recovery of the protein from cell culture. The polypeptide produced by a transformed cell can be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode cation transport 5 ATPase-like polypeptides can be designed to contain signal sequences which direct secretion of soluble cation transport ATPase-like polypeptides through a prokaryotic or eukaryotic cell membrane or which direct the membrane insertion of membrane-bound cation transport ATPase-like polypeptide. See WO 01/98340.

10 *Chemical synthesis*

Sequences encoding a human cation transport ATPase-like polypeptide can be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers *et al.*, *Nucl. Acids Res. Symp. Ser.* 215-223, 1980; Horn *et al.* *Nucl. Acids 15 Res. Symp. Ser.* 225-232, 1980). Alternatively, a human cation transport ATPase-like polypeptide itself can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques (Merrifield, *J. Am. Chem. Soc.* 85, 2149-2154, 1963; Roberge *et al.*, *Science* 269, 202-204, 1995). Protein synthesis can be performed using manual techniques or by 20 automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of cation transport ATPase-like polypeptides can be separately synthesized and combined using chemical methods to produce a full-length molecule. See WO 01/98340.

25

As will be understood by those of skill in the art, it may be advantageous to produce cation transport ATPase-like polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein 30 expression or to produce an RNA transcript having desirable properties, such as a

half-life which is longer than that of a transcript generated from the naturally occurring sequence.

5 The nucleotide sequences disclosed herein can be engineered using methods generally known in the art to alter cation transport ATPase-like polypeptide-encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the polypeptide or mRNA product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides can be used to engineer the nucleotide 10 sequences. For example, site-directed mutagenesis can be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

15 Antibodies

Any type of antibody known in the art can be generated to bind specifically to an epitope of a human cation transport ATPase-like polypeptide. "Antibody" as used herein includes intact immunoglobulin molecules, as well as fragments thereof, such as Fab, F(ab')₂, and Fv, which are capable of binding an epitope of a human cation 20 transport ATPase-like polypeptide. Typically, at least 6, 8, 10, or 12 contiguous amino acids are required to form an epitope. However, epitopes which involve non-contiguous amino acids may require more, e.g., at least 15, 25, or 50 amino acids.

An antibody which specifically binds to an epitope of a human cation transport 25 ATPase-like polypeptide can be used therapeutically, as well as in immunochemical assays, such as Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immunoprecipitations, or other immunochemical assays known in the art. Various immunoassays can be used to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric 30 assays are well known in the art. Such immunoassays typically involve the

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measurement of complex formation between an immunogen and an antibody that specifically binds to the immunogen.

Typically, an antibody that specifically binds to a human cation transport ATPase-like polypeptide provides a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in an immunochemical assay. Preferably, antibodies that specifically bind to cation transport ATPase-like polypeptides do not detect other proteins in immunochemical assays and can immunoprecipitate a human cation transport ATPase-like polypeptide from solution.

10 See WO 01/98340.

Antisense oligonucleotides

15 Antisense oligonucleotides are nucleotide sequences that are complementary to a specific DNA or RNA sequence. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form complexes and block either transcription or translation. Preferably, an antisense oligonucleotide is at least 11 nucleotides in length, but can be at least 12, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides long. Longer sequences also can be used. Antisense 20 oligonucleotide molecules can be provided in a DNA construct and introduced into a cell as described above to decrease the level of cation transport ATPase-like gene products in the cell.

25 Antisense oligonucleotides can be deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer, by covalently linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such alkyl-phosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamide, 30 carboxymethyl esters, carbonates, and phosphate triesters. See Brown, *Meth. Mol.*

Biol. 20, 1-8, 1994; Sonveaux, *Meth. Mol. Biol.* 26, 1-72, 1994; Uhlmann *et al.*, *Chem. Rev.* 90, 543-583, 1990.

Modifications of cation transport ATPase-like gene expression can be obtained by 5 designing antisense oligonucleotides that will form duplexes to the control, 5', or regulatory regions of the cation transport ATPase-like gene. Oligonucleotides derived from the transcription initiation site, *e.g.*, between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple 10 helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or chaperons. Therapeutic advances using triplex DNA have been described in the literature (*e.g.*, Gee *et al.*, in Huber & Carr, MOLECULAR AND IMMUNOLOGIC APPROACHES, Futura Publishing Co., Mt. Kisco, N.Y., 1994). An antisense oligonucleotide also can be designed to block translation 15 of mRNA by preventing the transcript from binding to ribosomes. See WO 01/98340.

Ribozymes

20 Ribozymes are RNA molecules with catalytic activity. See, *e.g.*, Cech, *Science* 236, 1532-1539; 1987; Cech, *Ann. Rev. Biochem.* 59, 543-568; 1990, Cech, *Curr. Opin. Struct. Biol.* 2, 605-609; 1992, Couture & Stinchcomb, *Trends Genet.* 12, 510-515, 1996. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art (*e.g.*, Haseloff *et al.*, U.S. Patent 5,641,673). The mechanism 25 of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of specific nucleotide sequences.

The coding sequence of a human cation transport ATPase-like polynucleotide can be used to generate ribozymes that will specifically bind to mRNA transcribed from the cation transport ATPase-like polynucleotide. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art (see Haseloff *et al.* 5 *Nature* 334, 585-591, 1988). For example, the cleavage activity of ribozymes can be targeted to specific RNAs by engineering a discrete "hybridization" region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target (see, for example, Gerlach *et* 10 *al.*, EP 321,201). See WO 01/98340.

Differentially expressed genes

Described herein are methods for the identification of genes whose products interact 15 with human cation transport ATPase-like protein. Such genes may represent genes that are differentially expressed in disorders including, but not limited to, CNS disorders, cardiovascular disorders, cancer, genitourinary disorders, and hematological disorders. Further, such genes may represent genes that are differentially regulated in response to manipulations relevant to the progression or treatment of 20 such diseases. Additionally, such genes may have a temporally modulated expression, increased or decreased at different stages of tissue or organism development. A differentially expressed gene may also have its expression modulated under control versus experimental conditions. In addition, the human cation transport ATPase-like gene or gene product may itself be tested for differential 25 expression.

The degree to which expression differs in a normal versus a diseased state need only be large enough to be visualized via standard characterization techniques such as differential display techniques. Other such standard characterization techniques by 30 which expression differences may be visualized include but are not limited to, quantitative RT (reverse transcriptase), PCR, and Northern analysis.

To identify differentially expressed genes total RNA or, preferably, mRNA is isolated from tissues of interest. For example, RNA samples are obtained from tissues of experimental subjects and from corresponding tissues of control subjects.

5 Any RNA isolation technique that does not select against the isolation of mRNA may be utilized for the purification of such RNA samples. See, for example, Ausubel *et al.*, ed., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, Inc. New York, 1987-1993. Large numbers of tissue samples may readily be processed using techniques well known to those of skill in the art, such as, for example, the

10 single-step RNA isolation process of Chomczynski, U.S. Patent 4,843,155.

Transcripts within the collected RNA samples that represent RNA produced by differentially expressed genes are identified by methods well known to those of skill in the art. They include, for example, differential screening (Tedder *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 85, 208-12, 1988), subtractive hybridization (Hedrick *et al.*, *Nature* 308, 149-53; Lee *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 88, 2825, 1984), and, preferably, differential display (Liang & Pardee, *Science* 257, 967-71, 1992; U.S. Patent 5,262,311).

20 The differential expression information may itself suggest relevant methods for the treatment of disorders involving the human cation transport ATPase-like protein. For example, treatment may include a modulation of expression of the differentially expressed genes and/or the gene encoding the human cation transport ATPase-like protein. The differential expression information may indicate whether the expression or activity of the differentially expressed gene or gene product or the human cation

25 transport ATPase-like gene or gene product are up-regulated or down-regulated.

Screening methods

30 The invention provides assays for screening test compounds that bind to or modulate the activity of a human cation transport ATPase-like polypeptide or a human cation

5 transport ATPase-like polynucleotide. A test compound preferably binds to a human cation transport ATPase-like polypeptide or polynucleotide. More preferably, a test compound decreases or increases enzymatic activity by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the test compound.

Test compounds

10 Test compounds can be pharmacologic agents already known in the art or can be compounds previously unknown to have any pharmacological activity. The compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be produced recombinantly, or synthesized by chemical methods known in the art. If desired, test compounds can be obtained using any of the numerous combinatorial library 15 methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches 20 are applicable to polypeptide, non-peptide oligomer, or small molecule libraries of compounds. *See Lam, Anticancer Drug Des.* 12, 145, 1997.

25 Methods for the synthesis of molecular libraries are well known in the art (see, for example, DeWitt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* '90, 6909, 1993; Erb *et al.* *Proc. Natl. Acad. Sci. U.S.A.* 91, 11422, 1994; Zuckermann *et al.*, *J. Med. Chem.* 37, 2678, 1994; Cho *et al.*, *Science* 261, 1303, 1993; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33, 2059, 1994; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33, 2061; Gallop *et al.*, *J. Med. Chem.* 37, 1233, 1994). Libraries of compounds can be presented in solution 30 (see, e.g., Houghten, *BioTechniques* 13, 412-421, 1992), or on beads (Lam, *Nature* 354, 82-84, 1991), chips (Fodor, *Nature* 364, 555-556, 1993), bacteria or spores (Ladner, U.S. Patent 5,223,409), plasmids (Cull *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*

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89, 1865-1869, 1992), or phage (Scott & Smith, *Science* 249, 386-390, 1990; Devlin, *Science* 249, 404-406, 1990); Cwirla *et al.*, *Proc. Natl. Acad. Sci.* 97, 6378-6382, 1990; Felici, *J. Mol. Biol.* 222, 301-310, 1991; and Ladner, U.S. Patent 5,223,409).

5 *High through-put screening*

Test compounds can be screened for the ability to bind to cation transport ATPase-like polypeptides or polynucleotides or to affect cation transport ATPase-like protein activity or cation transport ATPase-like gene expression using high throughput screening. Using high throughput screening, many discrete compounds can be tested in parallel so that large numbers of test compounds can be quickly screened. The most widely established techniques utilize 96-well microtiter plates. The wells of the microtiter plates typically require assay volumes that range from 50 to 500 μ l. In addition to the plates, many instruments, materials, pipettors, robotics, plate washers, 10 and plate readers are commercially available to fit the 96-well format.

15 Alternatively, "free format assays," or assays that have no physical barrier between samples, can be used. For example, an assay using pigment cells (melanocytes) in a simple homogeneous assay for combinatorial peptide libraries is described by Jayawickreme *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 91, 1614-18 (1994). The cells are placed under agarose in petri dishes, then beads that carry combinatorial compounds 20 are placed on the surface of the agarose. The combinatorial compounds are partially released the compounds from the beads. Active compounds can be visualized as dark pigment areas because, as the compounds diffuse locally into the gel matrix, the active compounds cause the cells to change colors.

25 Another example of a free format assay is described by Chelsky, "Strategies for Screening Combinatorial Libraries: Novel and Traditional Approaches," reported at the First Annual Conference of The Society for Biomolecular Screening in Philadelphia, Pa. (Nov. 7-10, 1995). Chelsky placed a simple homogenous enzyme assay for carbonic anhydrase inside an agarose gel such that the enzyme in the gel

would cause a color change throughout the gel. Thereafter, beads carrying combinatorial compounds via a photolinker were placed inside the gel and the compounds were partially released by UV-light. Compounds that inhibited the enzyme were observed as local zones of inhibition having less color change.

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Yet another example is described by Salmon *et al.*, *Molecular Diversity* 2, 57-63 (1996). In this example, combinatorial libraries were screened for compounds that had cytotoxic effects on cancer cells growing in agar.

10 Another high throughput screening method is described in Beutel *et al.*, U.S. Patent 5,976,813. In this method, test samples are placed in a porous matrix. One or more assay components are then placed within, on top of, or at the bottom of a matrix such as a gel, a plastic sheet, a filter, or other form of easily manipulated solid support. When samples are introduced to the porous matrix they diffuse sufficiently slowly, 15 such that the assays can be performed without the test samples running together.

Binding assays

20 For binding assays, the test compound is preferably a small molecule that binds to and occupies, for example, the active site of the cation transport ATPase-like polypeptide, such that normal biological activity is prevented. Examples of such small molecules include, but are not limited to, small peptides or peptide-like molecules.

25 In binding assays, either the test compound or the cation transport ATPase-like polypeptide can comprise a detectable label, such as a fluorescent, radioisotopic, chemiluminescent, or enzymatic label, such as horseradish peroxidase, alkaline phosphatase, or luciferase. Detection of a test compound that is bound to the cation transport ATPase-like polypeptide can then be accomplished, for example, by direct 30 counting of radioemission, by scintillation counting, or by determining conversion of an appropriate substrate to a detectable product.

Alternatively, binding of a test compound to a human cation transport ATPase-like polypeptide can be determined without labeling either of the interactants. For example, a microphysiometer can be used to detect binding of a test compound with 5 a human cation transport ATPase-like polypeptide. A microphysiometer (e.g., CytosensorTM) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a test compound and a human cation transport ATPase-like polypeptide 10 (McConnell *et al.*, *Science* 257, 1906-1912, 1992).

Determining the ability of a test compound to bind to a human cation transport ATPase-like polypeptide also can be accomplished using a technology such as real-time Bimolecular Interaction Analysis (BIA) (Sjolander & Urbaniczky, *Anal. Chem.* 63, 2338-2345, 1991, and Szabo *et al.*, *Curr. Opin. Struct. Biol.* 5, 699-705, 15 1995). BIA is a technology for studying biospecific interactions in real time, without labeling any of the interactants (e.g., BIACoreTM). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

20 In yet another aspect of the invention, a human cation transport ATPase-like polypeptide can be used as a "bait protein" in a two-hybrid assay or three-hybrid assay (see, e.g., U.S. Patent 5,283,317; Zervos *et al.*, *Cell* 72, 223-232, 1993; Madura *et al.*, *J. Biol. Chem.* 268, 12046-12054, 1993; Bartel *et al.*, *BioTechniques* 14, 25 920-924, 1993; Iwabuchi *et al.*, *Oncogene* 8, 1693-1696, 1993; and Brent W094/10300), to identify other proteins which bind to or interact with the cation transport ATPase-like polypeptide and modulate its activity.

30 The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. For example, in one construct, polynucleotide

encoding a human cation transport ATPase-like polypeptide can be fused to a polynucleotide encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct a DNA sequence that encodes an unidentified protein ("prey" or "sample") can be fused to a polynucleotide that codes for the 5 activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact *in vivo* to form an protein-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ), which is operably linked to a transcriptional regulatory site responsive to the 10 transcription factor. Expression of the reporter gene can be detected, and cell colonies containing the functional transcription factor can be isolated and used to obtain the DNA sequence encoding the protein that interacts with the cation transport ATPase-like polypeptide.

15 It may be desirable to immobilize either the cation transport ATPase-like polypeptide (or polynucleotide) or the test compound to facilitate separation of bound from unbound forms of one or both of the interactants, as well as to accommodate automation of the assay. Thus, either the cation transport ATPase-like polypeptide (or polynucleotide) or the test compound can be bound to a solid support. Suitable 20 solid supports include, but are not limited to, glass or plastic slides, tissue culture plates, microtiter wells, tubes, silicon chips, or particles such as beads (including, but not limited to, latex, polystyrene, or glass beads). Any method known in the art can be used to attach the enzyme polypeptide (or polynucleotide) or test compound to a solid support, including use of covalent and non-covalent linkages, passive 25 absorption, or pairs of binding moieties attached respectively to the polypeptide (or polynucleotide) or test compound and the solid support. Test compounds are preferably bound to the solid support in an array, so that the location of individual test compounds can be tracked. Binding of a test compound to a human cation transport ATPase-like polypeptide (or polynucleotide) can be accomplished in any 30 vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and microcentrifuge tubes.

In one embodiment, the cation transport ATPase-like polypeptide is a fusion protein comprising a domain that allows the cation transport ATPase-like polypeptide to be bound to a solid support. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and the non-adsorbed cation transport ATPase-like polypeptide; the mixture is then incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components. Binding of the interactants can be determined either directly or indirectly, as described above. Alternatively, the complexes can be dissociated from the solid support before binding is determined.

Other techniques for immobilizing proteins or polynucleotides on a solid support also can be used in the screening assays of the invention. For example, either a human cation transport ATPase-like polypeptide (or polynucleotide) or a test compound can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated cation transport ATPase-like polypeptides (or polynucleotides) or test compounds can be prepared from biotin-NHS(N- hydroxysuccinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, Ill.) and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies which specifically bind to a cation transport ATPase-like polypeptide, polynucleotide, or a test compound, but which do not interfere with a desired binding site, such as the active site of the cation transport ATPase-like polypeptide, can be derivatized to the wells of the plate. Unbound target or protein can be trapped in the wells by antibody conjugation.

Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies which specifically bind to the cation transport ATPase-like polypeptide or

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test compound, enzyme-linked assays which rely on detecting an activity of the cation transport ATPase-like polypeptide, and SDS gel electrophoresis under non-reducing conditions.

5 Screening for test compounds which bind to a human cation transport ATPase-like polypeptide or polynucleotide also can be carried out in an intact cell. Any cell which comprises a cation transport ATPase-like polypeptide or polynucleotide can be used in a cell-based assay system. A cation transport ATPase-like polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as
10 those described above. Binding of the test compound to a cation transport ATPase-like polypeptide or polynucleotide is determined as described above.

Enzymatic activity

15 Test compounds can be tested for the ability to increase or decrease the enzymatic activity of a human cation transport ATPase-like polypeptide. Enzymatic activity can be measured, for example, as described in J Biochem Mol Toxicol 1999; 13(2):93-106.
20 Enzyme assays can be carried out after contacting either a purified cation transport ATPase-like polypeptide, a cell membrane preparation, or an intact cell with a test compound. A test compound that decreases enzymatic activity of a human cation transport ATPase-like polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for
25 decreasing cation transport ATPase-like protein activity. A test compound which increases enzymatic activity of a human cation transport ATPase-like polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for increasing human cation transport ATPase-like protein activity.

Gene expression

In another embodiment, test compounds that increase or decrease cation transport ATPase-like gene expression are identified. A cation transport ATPase-like polynucleotide is contacted with a test compound, and the expression of an RNA or polypeptide product of the cation transport ATPase-like polynucleotide is determined. The level of expression of appropriate mRNA or polypeptide in the presence of the test compound is compared to the level of expression of mRNA or polypeptide in the absence of the test compound. The test compound can then be identified as a modulator of expression based on this comparison. For example, when expression of mRNA or polypeptide is greater in the presence of the test compound than in its absence, the test compound is identified as a stimulator or enhancer of the mRNA or polypeptide expression. Alternatively, when expression of the mRNA or polypeptide is less in the presence of the test compound than in its absence, the test compound is identified as an inhibitor of the mRNA or polypeptide expression.

The level of cation transport ATPase-like mRNA or polypeptide expression in the cells can be determined by methods well known in the art for detecting mRNA or polypeptide. Either qualitative or quantitative methods can be used. The presence of polypeptide products of a human cation transport ATPase-like polynucleotide can be determined, for example, using a variety of techniques known in the art, including immunochemical methods such as radioimmunoassay, Western blotting, and immunohistochemistry. Alternatively, polypeptide synthesis can be determined *in vivo*, in a cell culture, or in an *in vitro* translation system by detecting incorporation of labeled amino acids into a human cation transport ATPase-like polypeptide.

Such screening can be carried out either in a cell-free assay system or in an intact cell. Any cell that expresses a human cation transport ATPase-like polynucleotide can be used in a cell-based assay system. The cation transport ATPase-like polynucleotide can be naturally occurring in the cell or can be introduced using

techniques such as those described above. Either a primary culture or an established cell line, such as CHO or human embryonic kidney 293 cells, can be used.

Pharmaceutical compositions

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The invention also provides pharmaceutical compositions that can be administered to a patient to achieve a therapeutic effect. Pharmaceutical compositions of the invention can comprise, for example, a human cation transport ATPase-like polypeptide, cation transport ATPase-like polynucleotide, ribozymes or antisense oligonucleotides, antibodies which specifically bind to a cation transport ATPase-like polypeptide, or mimetics, activators, or inhibitors of a human cation transport ATPase-like polypeptide activity. The compositions can be administered alone or in combination with at least one other agent, such as stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions can be administered to a patient alone, or in combination with other agents, drugs or hormones.

20

In addition to the active ingredients, these pharmaceutical compositions can contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations which can be used pharmaceutically. Pharmaceutical compositions of the invention can be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, 25 subcutaneous, intraperitoneal, intranasal, parenteral, topical, sublingual, or rectal means. Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, 30 suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents can be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores can be used in conjunction with suitable coatings, such as concentrated sugar solutions, which also can contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, *i.e.*, dosage.

Pharmaceutical preparations that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds can be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations suitable for parenteral administration can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions can contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Addition-

ally, suspensions of the active compounds can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers also can be used for delivery.

5 Optionally, the suspension also can contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

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The pharmaceutical compositions of the present invention can be manufactured in a manner that is known in the art, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. The pharmaceutical composition can be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation can be a lyophilized powder which can contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% 15 mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

20

Further details on techniques for formulation and administration can be found in the latest edition of REMINGTON'S PHARMACEUTICAL SCIENCES (Maack Publishing Co., Easton, Pa.). After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated 25 condition. Such labeling would include amount, frequency, and method of administration.

Therapeutic indications and methods

Human cation transport ATPase-like protein can be regulated to treat CNS disorders, cardiovascular disorders, cancer, genitourinary disorders, and hematological disorders.

Central Nervous System (CNS) Disorders

The novel human cation transport ATPase-like protein of the invention is highly expressed in the following brain tissues: cerebellum, cerebellum (left), Alzheimer cerebral cortex, cerebellum (right), tonsilla cerebelli, postcentral gyrus, vermis cerebelli, parietal lobe, cerebral peduncles, precentral gyrus, occipital lobe, Alzheimer brain frontal lobe, hippocampus, cerebral cortex, temporal lobe, frontal lobe, retina, Alzheimer brain, pons, brain, thalamus, spinal cord, corpus callosum, neuroblastoma IMR32 cells, cerebral meninges, and dorsal root ganglia. The expression in brain tissues and in particular the differential expression between diseased tissue (Alzheimer cerebral cortex, Alzheimer brain frontal lobe) and healthy tissue (cerebral cortex, frontal lobe), demonstrates that the novel human cation transport ATPase-like protein or mRNA can be utilized to diagnose nervous system diseases. Additionally the activity of the novel human cation transport ATPase-like protein can be modulated to treat nervous system diseases.

CNS disorders include disorders of the central nervous system as well as disorders of the peripheral nervous system. CNS disorders include, but are not limited to, brain injuries, cerebrovascular diseases and their consequences, Parkinson's disease, corticobasal degeneration, motor neuron disease (including ALS), multiple sclerosis, traumatic brain injury, stroke, post-stroke, post-traumatic brain injury, and small-vessel cerebrovascular disease. Dementias, such as Alzheimer's disease, vascular dementia, dementia with Lewy bodies, frontotemporal dementia and Parkinsonism linked to chromosome 17, frontotemporal dementias (including Pick's disease), progressive nuclear palsy, corticobasal degeneration, Huntington's disease, thalamic

degeneration, Creutzfeld-Jakob dementia, HIV dementia, schizophrenia with dementia, and Korsakoff's psychosis, also are CNS disorders.

5 Similarly, cognitive-related disorders, such as mild cognitive impairment, age-associated memory impairment, age-related cognitive decline, vascular cognitive impairment, attention deficit disorders, attention deficit hyperactivity disorders, and memory disturbances in children with learning disabilities also are considered to be CNS disorders.

10 Pain, within the meaning of the invention, is also considered to be a CNS disorder. Pain can be associated with CNS disorders, such as multiple sclerosis, spinal cord injury, sciatica, failed back surgery syndrome, traumatic brain injury, epilepsy, Parkinson's disease, post-stroke, and vascular lesions in the brain and spinal cord (e.g., infarct, hemorrhage, vascular malformation). Non-central neuropathic pain 15 includes that associated with post mastectomy pain, phantom feeling, reflex sympathetic dystrophy (RSD), trigeminal neuralgia/radioculopathy, post-surgical pain, HIV/AIDS related pain, cancer pain, metabolic neuropathies (e.g., diabetic neuropathy, vasculitic neuropathy secondary to connective tissue disease), paraneoplastic polyneuropathy associated, for example, with carcinoma of lung, or 20 leukemia, or lymphoma, or carcinoma of prostate, colon or stomach, trigeminal neuralgia, cranial neuralgias, and post-herpetic neuralgia. Pain is also associated with peripheral nerve damage, central pain (e.g., due to cerebral ischemia) and various chronic pain (e.g., lumbago, back pain (low back pain), inflammatory and/or rheumatic pain. Headache pain (for example, migraine with aura, migraine without 25 aura, and other migraine disorders), episodic and chronic tension-type headache, tension-type like headache, cluster headache, and chronic paroxysmal hemicrania also are CNS disorders. Visceral pain, such as pancreatitis, intestinal cystitis, dysmenorrhea, irritable Bowel syndrome, Crohn's disease, biliary colic, ureteral colic, myocardial infarction and pain syndromes of the pelvic cavity, e.g., 30 vulvodynia, orchialgia, urethral syndrome and protatodynia also is a CNS disorder.

Also considered to be disorders of the nervous system are acute pain, for example postoperative pain, and pain after trauma.

Cardiovascular Disorders

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The novel human cation transport ATPase-like protein is highly expressed in the following cardiovascular related tissues: aorta, artery, heart ventricle (left), and vein. Expression in the above mentioned tissues demonstrates that the novel human cation transport ATPase-like protein or mRNA can be utilized to diagnose of cardiovascular 10 diseases. Additionally the activity of the novel human cation transport ATPase-like protein can be modulated to treat cardiovascular diseases.

15 Cardiovascular diseases include the following disorders of the heart and the vascular system: congestive heart failure, myocardial infarction, ischemic diseases of the heart, all kinds of atrial and ventricular arrhythmias, hypertensive vascular diseases, and peripheral vascular diseases.

20 Heart failure is defined as a pathophysiologic state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirement of the metabolizing tissue. It includes all forms of pumping failure, such as high-output and low-output, acute and chronic, right-sided or left-sided, systolic or diastolic, independent of the underlying cause.

25 Myocardial infarction (MI) is generally caused by an abrupt decrease in coronary blood flow that follows a thrombotic occlusion of a coronary artery previously narrowed by arteriosclerosis. MI prophylaxis (primary and secondary prevention) is included, as well as the acute treatment of MI and the prevention of complications.

30 Ischemic diseases are conditions in which the coronary flow is restricted resulting in a perfusion which inadequate to meet the myocardial requirement for oxygen. This

group of diseases includes stable angina, unstable angina, and asymptomatic ischemia.

5 Arrhythmias include all forms of atrial and ventricular tachyarrhythmias (atrial tachycardia, atrial flutter, atrial fibrillation, atrio-ventricular reentrant tachycardia, preexcitation syndrome, ventricular tachycardia, ventricular flutter, and ventricular fibrillation), as well as bradycardic forms of arrhythmias.

10 Vascular diseases include primary as well as all kinds of secondary arterial hypertension (renal, endocrine, neurogenic, others). The disclosed gene and its product may be used as drug targets for the treatment of hypertension as well as for the prevention of all complications. Peripheral vascular diseases are defined as vascular diseases in which arterial and/or venous flow is reduced resulting in an imbalance between blood supply and tissue oxygen demand. It includes chronic peripheral 15 arterial occlusive disease (PAOD), acute arterial thrombosis and embolism, inflammatory vascular disorders, Raynaud's phenomenon, and venous disorders.

Genitourinary disorders

20 The novel human cation transport ATPase-like protein is highly expressed in the following tissues of the genitourinary system: testis, ovary tumor, penis, prostate, and prostate BPH. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue (prostate BPH) and healthy tissue (prostate) demonstrates that the novel human cation transport ATPase-like protein or 25 mRNA can be utilized to diagnose of genitourinary disorders. Additionally the activity of the novel human cation transport ATPase-like protein can be modulated to treat genitourinary disorders.

30 Geniturological disorders comprise benign and malign disorders of the organs constituting the geniturological system of female and male, renal diseases such as acute or chronic renal failure, immunologically mediated renal diseases such as renal

transplant rejection, lupus nephritis, immune complex renal diseases, glomerulopathies, nephritis, toxic nephropathy, obstructive uropathies such as benign prostatic hyperplasia (BPH), neurogenic bladder syndrome, urinary incontinence such as urge-, stress-, or overflow incontinence, pelvic pain, and erectile dysfunction.

5

Cancer Disorders

10 The novel human cation transport ATPase-like protein is highly expressed in the following cancer tissues: ovarian tumor and lung tumor. The expression in the above mentioned tissues and in particular the differential expression between diseased tissue (tumor) and healthy tissue demonstrates that the novel human cation transport ATPase-like protein or mRNA can be utilized to diagnose of cancer. Additionally the activity of the novel human cation transport ATPase-like protein can be modulated to treat cancer.

15

20 Cancer disorders within the scope of the invention comprise any disease of an organ or tissue in mammals characterized by poorly controlled or uncontrolled multiplication of normal or abnormal cells in that tissue and its effect on the body as a whole. Cancer diseases within the scope of the invention comprise benign neoplasms; dysplasias, hyperplasias as well as neoplasms showing metastatic growth or any other transformations, e.g., leukoplakias, which often precede a breakout of cancer. Cells and tissues are cancerous when they grow more rapidly than normal cells, displacing or spreading into the surrounding healthy tissue or any other tissues of the body described as metastatic growth, assume abnormal shapes and sizes, show 25 changes in their nucleocytoplasmatic ratio, nuclear polychromasia, and finally may cease.

30 Cancerous cells and tissues may affect the body as a whole when causing paraneoplastic syndromes or if cancer occurs within a vital organ or tissue, normal function will be impaired or halted, with possible fatal results. The ultimate involvement of a vital organ by cancer, either primary or metastatic, may lead to the death of the

mammal affected. Cancer tends to spread, and the extent of its spread is usually related to an individual's chances of surviving the disease. Cancers are generally said to be in one of three stages of growth: early, or localized, when a tumor is still confined to the tissue of origin, or primary site; direct extension, where cancer cells 5 from the tumour have invaded adjacent tissue or have spread only to regional lymph nodes; or metastasis, in which cancer cells have migrated to distant parts of the body from the primary site, via the blood or lymph systems, and have established secondary sites of infection. Cancer is said to be malignant because of its tendency to cause death if not treated.

10

Benign tumors usually do not cause death, although they may if they interfere with a normal body function by virtue of their location, size, or paraneoplastic side effects. Hence, benign tumors fall under the definition of cancer within the scope of the invention as well. In general, cancer cells divide at a higher rate than do normal cells, 15 but the distinction between the growth of cancerous and normal tissues is not so much the rapidity of cell division in the former as it is the partial or complete loss of growth restraint in cancer cells and their failure to differentiate into a useful, limited tissue of the type that characterizes the functional equilibrium of growth of normal tissue.

20

Cancer tissues may express certain molecular receptors and probably are influenced by the host's susceptibility and immunity and it is known that certain cancers of the breast and prostate, for example, are considered dependent on specific hormones for their existence. The term "cancer" under the scope of the invention is not limited to 25 simple benign neoplasia but includes any other benign and malign neoplasia, such as 1) carcinoma, 2) sarcoma, 3) carcinosarcoma, 4) cancers of the blood-forming tissues, 5) tumors of nerve tissues including the brain, and 6) cancer of skin cells.

30

Carcinoma occurs in epithelial tissues, which cover the outer body (the skin) and line mucous membranes and the inner cavitary structures of organs e.g. such as the breast, lung, the respiratory and gastrointestinal tracts, the endocrine glands, and the

5 genitourinary system. Ductal or glandular elements may persist in epithelial tumors, as in adenocarcinomas, e.g., thyroid adenocarcinoma, gastric adenocarcinoma, uterine adenocarcinoma. Cancers of the pavement-cell epithelium of the skin and of certain mucous membranes, such as cancers of the tongue, lip, larynx, urinary bladder, uterine cervix, or penis, may be termed epidermoid or squamous-cell carcinomas of the respective tissues and are within the scope of the definition of cancer as well.

10 Sarcomas develop in connective tissues, including fibrous tissues, adipose (fat) tissues, muscle, blood vessels, bone, and cartilage such as osteogenic sarcoma, liposarcoma, fibrosarcoma, and synovial sarcoma.

15 Carcinosarcoma is cancer that develops in both epithelial and connective tissue. Cancer disease within the scope of this definition may be primary or secondary, whereby primary indicates that the cancer originated in the tissue where it is found rather than was established as a secondary site through metastasis from another lesion. Cancers and tumor diseases within the scope of this definition may be benign or malign and may affect all anatomical structures of the body of a mammal. By example, to they comprise cancers and tumor diseases of I) the bone marrow and 20 bone marrow derived cells (leukemias), II) the endocrine and exocrine glands, such as the thyroid, parathyroid, pituitary, adrenal glands, salivary glands, and pancreas III) the breast, such as benign or malignant tumors in the mammary glands of either a male or a female, the mammary ducts, adenocarcinoma, medullary carcinoma, comedocarcinoma, Paget's disease of the nipple, inflammatory carcinoma of the 25 young woman, IV) the lung, V) the stomach, VI) the liver and spleen, VII) the small intestine, VIII) the colon, IX) the bone and its supportive and connective tissues such as malignant or benign bone tumour, such as malignant osteogenic sarcoma, benign osteoma, cartilage tumors, malignant chondrosarcoma or benign chondroma, bone marrow tumors such as malignant myeloma or benign eosinophilic granuloma, as 30 well as metastatic tumors from bone tissues at other locations of the body; X) the mouth, throat, larynx, and the esophagus, XI) the urinary bladder and the internal and

external organs and structures of the urogenital system of male and female such as the ovaries, uterus, cervix of the uterus, testes, and prostate gland, XII) the prostate, XIII) the pancreas, such as ductal carcinoma of the pancreas; XIV) the lymphatic tissue such as lymphomas and other tumors of lymphoid origin, XV) the skin, XVI) cancers and tumor diseases of all anatomical structures belonging to the respiratory systems including thoracal muscles and linings, XVII) primary or secondary cancer of the lymph nodes, XVIII) the tongue and of the bony structures of the hard palate or sinuses, XVIV) the mouth, cheeks, neck and salivary glands, XX) the blood vessels including the heart and their linings, XXI) the smooth or skeletal muscles and 5 their ligaments and linings, XXII) the peripheral, the autonomous, the central nervous system including the cerebellum, and XXIII) the adipose tissue.

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Hematological Disorders

15 The novel human cation transport ATPase-like protein is highly expressed in the following tissues of the hematological system: thrombocytes, lymph node, and bone marrow CD15⁺ cells. The expression in the above mentioned tissues demonstrates that the novel human cation transport ATPase-like protein or mRNA can be utilized to diagnose of hematological diseases. Additionally the activity of the novel human 20 cation transport ATPase-like protein can be modulated to treat hematological disorders.

Hematological disorders include diseases of the blood and all its constituents as well as diseases of organs involved in the generation or degradation of the blood. They 25 include but are not limited to 1) anemias, 2) myeloproliferative disorders, 3) hemorrhagic disorders, 4) leukopenia, 5) eosinophilic disorders, 6) leukemias, 7) lymphomas, 8) plasma cell dyscrasias, and 9) disorders of the spleen in the course of hematological disorders.

30 Anemias include, but are not limited to, anemias due to defective or deficient hem synthesis, deficient erythropoiesis. Myeloproliferative disorders include, but are not

limited to, polycythemia vera, tumor-associated erythrocytosis, myelofibrosis, thrombocythemia. Hemorrhagic disorders include, but are not limited to, vasculitis, thrombocytopenia, heparin-induced thrombocytopenia, thrombotic thrombocytopenic purpura, hemolytic-uremic syndrome, hereditary and acquired disorders of platelet 5 function, hereditary coagulation disorders. Leukopenias include, but are not limited to neutropenia and lymphocytopenia. Eosinophilic disorders include, but are not limited to hypereosinophilia and idiopathic hypereosinophilic syndrome. Leukemias include, but are not limited to, acute myeloid leukemia, acute lymphoblastic leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia, and 10 myelodysplastic syndrome. Lymphomas include, but are not limited to, Hodgkin's disease, non-Hodgkin's lymphoma, Burkett's lymphoma, mycosis fungoides cutaneous T-cell lymphoma. Plasma cell dyscrasias include, but are not limited to, multiple myeloma, macroglobulinemia, and heavy chain diseases. Idiopathic thrombocytopenic purpura, iron deficiency anemia, megaloblastic anemia (vitamin 15 B12 deficiency), aplastic anemia, thalassemia, malignant lymphoma bone marrow invasion, malignant lymphoma skin invasion, haemolytic uraemic syndrome, and giant platelet disease also are hematological diseases.

This invention further pertains to the use of novel agents identified by the screening 20 assays described above. Accordingly, it is within the scope of this invention to use a test compound identified as described herein in an appropriate animal model. For example, an agent identified as described herein (e.g., a modulating agent, an antisense nucleic acid molecule, a specific antibody, ribozyme, or a human cation transport ATPase-like polypeptide binding molecule) can be used in an animal model 25 to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

A reagent which affects cation transport ATPase-like protein activity can be administered to a human cell, either *in vitro* or *in vivo*, to reduce cation transport ATPase-like protein activity. The reagent preferably binds to an expression product of a human cation transport ATPase-like gene. If the expression product is a protein, 5 the reagent is preferably an antibody. For treatment of human cells *ex vivo*, an antibody can be added to a preparation of stem cells that have been removed from the body. The cells can then be replaced in the same or another human body, with or without clonal propagation, as is known in the art.

10 In one embodiment, the reagent is delivered using a liposome. Preferably, the liposome is stable in the animal into which it has been administered for at least about 30 minutes, more preferably for at least about 1 hour, and even more preferably for at least about 24 hours. A liposome comprises a lipid composition that is capable of targeting a reagent, particularly a polynucleotide, to a particular site in an animal, 15 such as a human. Preferably, the lipid composition of the liposome is capable of targeting to a specific organ of an animal, such as the lung, liver, spleen, heart brain, lymph nodes, and skin.

20 A liposome useful in the present invention comprises a lipid composition that is capable of fusing with the plasma membrane of the targeted cell to deliver its contents to the cell. Preferably, the transfection efficiency of a liposome is about 0.5 μ g of DNA per 16 nmole of liposome delivered to about 10^6 cells, more preferably about 1.0 μ g of DNA per 16 nmole of liposome delivered to about 10^6 cells, and even more preferably about 2.0 μ g of DNA per 16 nmol of liposome 25 delivered to about 10^6 cells. Preferably, a liposome is between about 100 and 500 nm, more preferably between about 150 and 450 nm, and even more preferably between about 200 and 400 nm in diameter.

30 Suitable liposomes for use in the present invention include those liposomes standardly used in, for example, gene delivery methods known to those of skill in the art. More preferred liposomes include liposomes having a polycationic lipid

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composition and/or liposomes having a cholesterol backbone conjugated to polyethylene glycol. Optionally, a liposome comprises a compound capable of targeting the liposome to a particular cell type, such as a cell-specific ligand exposed on the outer surface of the liposome.

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Complexing a liposome with a reagent such as an antisense oligonucleotide or ribozyme can be achieved using methods that are standard in the art (see, for example, U.S. Patent 5,705,151). Preferably, from about 0.1 μ g to about 10 μ g of polynucleotide is combined with about 8 nmol of liposomes, more preferably from 10 about 0.5 μ g to about 5 μ g of polynucleotides are combined with about 8 nmol liposomes, and even more preferably about 1.0 μ g of polynucleotides is combined with about 8 nmol liposomes.

15 In another embodiment, antibodies can be delivered to specific tissues *in vivo* using receptor-mediated targeted delivery. Receptor-mediated DNA delivery techniques are taught in, for example, Findeis *et al.* *Trends in Biotechnol.* 11, 202-05 (1993); Chiou *et al.*, *GENE THERAPEUTICS: METHODS AND APPLICATIONS OF DIRECT GENE TRANSFER* (J.A. Wolff, ed.) (1994); Wu & Wu, *J. Biol. Chem.* 263, 621-24 (1988); Wu *et al.*, *J. Biol. Chem.* 269, 542-46 (1994); Zenke *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 87, 3655-59 (1990); Wu *et al.*, *J. Biol. Chem.* 266, 338-42 (1991).

Determination of a therapeutically effective dose

25 The determination of a therapeutically effective dose is well within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of active ingredient which increases or decreases enzymatic activity relative to the enzymatic activity which occurs in the absence of the therapeutically effective dose.

30 For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model also can be used to determine the appropriate concentration range and

route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

Therapeutic efficacy and toxicity, e.g., ED₅₀ (the dose therapeutically effective in 5 50% of the population) and LD₅₀ (the dose lethal to 50% of the population), can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD₅₀/ED₅₀.

10 Pharmaceutical compositions that exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage varies within this range depending upon the dosage 15 form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active ingredient or to maintain the desired effect. 20 Factors that can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions can be administered every 3 to 4 days, every week, or once every two weeks depending on 25 the half-life and clearance rate of the particular formulation.

Normal dosage amounts can vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available 30 to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of poly-

nucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

If the reagent is a single-chain antibody, polynucleotides encoding the antibody can 5 be constructed and introduced into a cell either *ex vivo* or *in vivo* using well-established techniques including, but not limited to, transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, "gene gun," and DEAE- or calcium 10 phosphate-mediated transfection.

Effective *in vivo* dosages of an antibody are in the range of about 5 μ g to about 50 μ g/kg, about 50 μ g to about 5 mg/kg, about 100 μ g to about 500 μ g/kg of patient 15 body weight, and about 200 to about 250 μ g/kg of patient body weight. For administration of polynucleotides encoding single-chain antibodies, effective *in vivo* dosages are in the range of about 100 ng to about 200 ng, 500 ng to about 50 mg, about 1 μ g to about 2 mg, about 5 μ g to about 500 μ g, and about 20 μ g to about 100 μ g of DNA.

If the expression product is mRNA, the reagent is preferably an antisense 20 oligonucleotide or a ribozyme. Polynucleotides that express antisense oligonucleotides or ribozymes can be introduced into cells by a variety of methods, as described above.

25 Preferably, a reagent reduces expression of a human cation transport ATPase-like gene or the activity of a cation transport ATPase-like polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the reagent. The effectiveness of the mechanism chosen to decrease the level of expression of a human cation transport ATPase-like gene or the activity of a 30 human cation transport ATPase-like polypeptide can be assessed using methods well known in the art, such as hybridization of nucleotide probes to cation transport

ATPase-like protein-specific mRNA, quantitative RT-PCR, immunologic detection of a human cation transport ATPase-like polypeptide, or measurement of enzymatic activity.

5 In any of the embodiments described above, any of the pharmaceutical compositions of the invention can be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy can be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents can act synergistically to effect the
10 treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

15 Any of the therapeutic methods described above can be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

Diagnostic methods

20 Human cation transport ATPase-like protein also can be used in diagnostic assays for detecting diseases and abnormalities or susceptibility to diseases and abnormalities related to the presence of mutations in the nucleic acid sequences that encode the enzyme. For example, differences can be determined between the cDNA or genomic sequence encoding cation transport ATPase-like protein in individuals afflicted with
25 a disease and in normal individuals. If a mutation is observed in some or all of the afflicted individuals but not in normal individuals, then the mutation is likely to be the causative agent of the disease.

30 Sequence differences between a reference gene and a gene having mutations can be revealed by the direct DNA sequencing method. In addition, cloned DNA segments can be employed as probes to detect specific DNA segments. The sensitivity of this

method is greatly enhanced when combined with PCR. For example, a sequencing primer can be used with a double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures using radiolabeled nucleotides or by automatic sequencing procedures using fluorescent tags.

Genetic testing based on DNA sequence differences can be carried out by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized, for example, by high resolution gel electrophoresis. DNA fragments of different sequences can be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, *Science* 230, 1242, 1985). Sequence changes at specific locations can also be revealed by nuclease protection assays, such as RNase and S 1 protection or the chemical cleavage method (e.g., Cotton *et al.*, *Proc. Natl. Acad. Sci. USA* 85, 4397-4401, 1985). Thus, the detection of a specific DNA sequence can be performed by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes and Southern blotting of genomic DNA. In addition to direct methods such as gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

Altered levels of cation transport ATPase-like protein also can be detected in various tissues. Assays used to detect levels of the receptor polypeptides in a body sample, such as blood or a tissue biopsy, derived from a host are well known to those of skill in the art and include radioimmunoassays, competitive binding assays, Western blot analysis, and ELISA assays.

All patents and patent applications cited in this disclosure are expressly incorporated herein by reference. The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following

- 50 -

specific examples, which are provided for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLE 1

5

Detection of cation transport ATPase-like protein activity

The polynucleotide of SEQ ID NO: 3 is inserted into the expression vector pCEV4 and the expression vector pCEV4-cation transporting ATPase-like protein polypeptide obtained is transfected into human embryonic kidney 293 cells. From these 10 cells extracts are obtained and ATPase-like protein activity is measured as the liberation of 32Pi from [γ -32P]ATP. In general, samples are preincubated with or without 2.5 μ g of designated phospholipid, for 10 min at 25°C. ATPase-like protein assay is started by the addition of 0.2 ml of assay solution consisting of 15 50 mM Tris-MES; pH 7.0, 30 mM KCl, 3 mM MgCl₂, and 3-5 mM [γ -³²P]ATP (400 cpm/nmol), with or without 0.1 mM sodium vanadate as an inhibitor, and incubated at 37°C for 30 min. The reaction is terminated by the addition of 1 ml of 1.25 N perchloric acid, and phosphate is extracted and counted in 20 a scintillation counter. The results are expressed as nmol of Pi/min/assay, or calculated as μ mol of Pi/min/mg of protein. It is shown that the polypeptide of SEQ ID NO: 2 has a cation transporting ATPase-like protein activity.

EXAMPLE 2

25 *Expression of recombinant human cation transport ATPase-like protein*

The *Pichia pastoris* expression vector pPICZB (Invitrogen, San Diego, CA) is used to produce large quantities of recombinant human cation transport ATPase-like polypeptides in yeast. The cation transport ATPase-like protein-encoding DNA 30 sequence is derived from SEQ ID NO: 1. Before insertion into vector pPICZB, the DNA sequence is modified by well known methods in such a way that it contains at

its 5'-end an initiation codon and at its 3'-end an enterokinase cleavage site, a His6 reporter tag and a termination codon. Moreover, at both termini recognition sequences for restriction endonucleases are added and after digestion of the multiple cloning site of pPICZ B with the corresponding restriction enzymes the modified 5. DNA sequence is ligated into pPICZB. This expression vector is designed for inducible expression in *Pichia pastoris*, driven by a yeast promoter. The resulting pPICZ/md-His6 vector is used to transform the yeast.

10 The yeast is cultivated under usual conditions in 5 liter shake flasks and the recombinantly produced protein isolated from the culture by affinity chromatography (Ni-NTA-Resin) in the presence of 8 M urea. The bound polypeptide is eluted with buffer, pH 3.5, and neutralized. Separation of the polypeptide from the His6 reporter tag is accomplished by site-specific proteolysis using enterokinase (Invitrogen, San 15 Diego, CA) according to manufacturer's instructions. Purified human cation transport ATPase-like polypeptide is obtained.

EXAMPLE 3

20 *Identification of test compounds that bind to cation transport ATPase-like polypeptides*

Purified cation transport ATPase-like polypeptides comprising a glutathione-S-transferase protein and absorbed onto glutathione-derivatized wells of 96-well microtiter plates are contacted with test compounds from a small molecule library at 25 pH 7.0 in a physiological buffer solution. Human cation transport ATPase-like polypeptides comprise the amino acid sequence shown in SEQ ID NO: 2. The test compounds comprise a fluorescent tag. The samples are incubated for 5 minutes to one hour. Control samples are incubated in the absence of a test compound.

30 The buffer solution containing the test compounds is washed from the wells. Binding of a test compound to a human cation transport ATPase-like polypeptide is

detected by fluorescence measurements of the contents of the wells. A test compound that increases the fluorescence in a well by at least 15% relative to fluorescence of a well in which a test compound is not incubated is identified as a compound which binds to a human cation transport ATPase-like polypeptide.

5

EXAMPLE 4

Identification of a test compound which decreases cation transport ATPase-like gene expression

10

A test compound is administered to a culture of human cells transfected with a cation transport ATPase-like protein expression construct and incubated at 37°C for 10 to 45 minutes. A culture of the same type of cells that have not been transfected is incubated for the same time without the test compound to provide a negative control.

15

RNA is isolated from the two cultures as described in Chirgwin *et al.*, *Biochem.* 18, 5294-99, 1979). Northern blots are prepared using 20 to 30 µg total RNA and hybridized with a ³²P-labeled cation transport ATPase-like protein-specific probe at 65°C in Express-hyb (CLONTECH). The probe comprises at least 11 contiguous nucleotides selected from the complement of SEQ ID NO: 1 or 3. A test compound that decreases the cation transport ATPase-like protein-specific signal relative to the signal obtained in the absence of the test compound is identified as an inhibitor of cation transport ATPase-like gene expression.

25

EXAMPLE 5

Identification of a test compound which decreases cation transport ATPase-like protein activity

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A test compound is administered to a culture of human cells transfected with a cation transport ATPase-like protein expression construct and incubated at 37°C for 10 to

45 minutes. A culture of the same type of cells that have not been transfected is incubated for the same time without the test compound to provide a negative control. Enzymatic activity is measured using the method of J Biochem Mol Toxicol 1999;13(2):93-106.

5

A test compound which decreases the enzymatic activity of the cation transport ATPase-like protein relative to the enzymatic activity in the absence of the test compound is identified as an inhibitor of cation transport ATPase-like protein activity.

10

EXAMPLE 6

Tissue-specific expression of cation transport ATPase-like protein

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The qualitative expression pattern of cation transport ATPase-like protein in various tissues is determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

20

Quantitative expression profiling is performed by the form of quantitative PCR analysis called "kinetic analysis" firstly described in Higuchi *et al.*, *BioTechnology* 10, 413-17, 1992, and Higuchi *et al.*, *BioTechnology* 11, 1026-30, 1993. The principle is that at any given cycle within the exponential phase of PCR, the amount of product is proportional to the initial number of template copies.

25

If the amplification is performed in the presence of an internally quenched fluorescent oligonucleotide (TaqMan probe) complementary to the target sequence, the probe is cleaved by the 5'-3' endonuclease activity of Taq DNA polymerase and a fluorescent dye released in the medium (Holland *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 88, 7276-80, 1991). Because the fluorescence emission will increase in direct proportion to the amount of the specific amplified product, the exponential growth phase of PCR product can be detected and used to determine the initial template

30

concentration (Heid *et al.*, *Genome Res.* 6, 986-94, 1996, and Gibson *et al.*, *Genome Res.* 6, 995-1001, 1996).

5 The amplification of an endogenous control can be performed to standardize the amount of sample RNA added to a reaction. In this kind of experiment, the control of choice is the 18S ribosomal RNA. Because reporter dyes with differing emission spectra are available, the target and the endogenous control can be independently quantified in the same tube if probes labeled with different dyes are used. All "real time PCR" measurements of fluorescence are made in the ABI Prism 7700.

10 *RNA extraction and cDNA preparation.* Total RNA from the tissues listed above are used for expression quantification. RNAs labeled "from autopsy" were extracted from autoptic tissues with the TRIzol reagent (Life Technologies, MD) according to the manufacturer's protocol.

15 Fifty µg of each RNA were treated with DNase I for 1 hour at 37°C in the following reaction mix: 0.2 U/µl RNase-free DNase I (Roche Diagnostics, Germany); 0.4 U/µl RNase inhibitor (PE Applied Biosystems, CA); 10 mM Tris-HCl pH 7.9; 10 mM MgCl₂; 50 mM NaCl; and 1 mM DTT.

20 After incubation, RNA is extracted once with 1 volume of phenol:chloroform:isoamyl alcohol (24:24:1) and once with chloroform, and precipitated with 1/10 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of ethanol.

25 Fifty µg of each RNA from the autoptic tissues are DNase treated with the DNA-free kit purchased from Ambion (Ambion, TX). After resuspension and spectrophotometric quantification, each sample is reverse transcribed with the TaqMan Reverse Transcription Reagents (PE Applied Biosystems, CA) according to the manufacturer's protocol. The final concentration of RNA in the reaction mix is 30 200 ng/µL. Reverse transcription is carried out with 2.5 µM of random hexamer primers.

5 *TaqMan quantitative analysis.* Specific primers and probe are designed according to the recommendations of PE Applied Biosystems; the probe can be labeled at the 5' end FAM (6-carboxy-fluorescein) and at the 3' end with TAMRA (6-carboxy-tetramethyl-rhodamine). Quantification experiments are performed on 10 ng of reverse transcribed RNA from each sample. Each determination is done in triplicate.

10 Total cDNA content is normalized with the simultaneous quantification (multiplex PCR) of the 18S ribosomal RNA using the Pre-Developed TaqMan Assay Reagents (PDAR) Control Kit (PE Applied Biosystems, CA).

15 The assay reaction mix is as follows: 1X final TaqMan Universal PCR Master Mix (from 2X stock) (PE Applied Biosystems, CA); 1X PDAR control – 18S RNA (from 20X stock); 300 nM forward primer; 900 nM reverse primer; 200 nM probe; 10 ng cDNA; and water to 25 μ l.

20 Each of the following steps are carried out once: pre PCR, 2 minutes at 50°C, and 10 minutes at 95°C. The following steps are carried out 40 times: denaturation, 15 seconds at 95°C, annealing/extension, 1 minute at 60°C.

25 The experiment is performed on an ABI Prism 7700 Sequence Detector (PE Applied Biosystems, CA). At the end of the run, fluorescence data acquired during PCR are processed as described in the ABI Prism 7700 user's manual in order to achieve better background subtraction as well as signal linearity with the starting target quantity.

EXAMPLE 7

Proliferation inhibition assay: Antisense oligonucleotides suppress the growth of cancer cell lines

5

The cell line used for testing is the human colon cancer cell line HCT116. Cells are cultured in RPMI-1640 with 10-15% fetal calf serum at a concentration of 10,000 cells per milliliter in a volume of 0.5 ml and kept at 37°C in a 95% air/5%CO₂ atmosphere.

10

Phosphorothioate oligoribonucleotides are synthesized on an Applied Biosystems Model 380B DNA synthesizer using phosphoroamidite chemistry. A sequence of 24 bases complementary to the nucleotides at position 1 to 24 of SEQ ID NO: 1 is used as the test oligonucleotide. As a control, another (random) sequence is used: 5'-TCA 15 ACT GAC TAG ATG TAC ATG GAC-3' (SEQ ID NO: 5). Following assembly and deprotection, oligonucleotides are ethanol-precipitated twice, dried, and suspended in phosphate buffered saline at the desired concentration. Purity of the oligonucleotides is tested by capillary gel electrophoresis and ion exchange HPLC. The purified oligonucleotides are added to the culture medium at a concentration of 20 10 µM once per day for seven days.

25

The addition of the test oligonucleotide for seven days results in significantly reduced expression of human cation transport ATPase-like protein as determined by Western blotting. This effect is not observed with the control oligonucleotide. After 3 to 7 days, the number of cells in the cultures is counted using an automatic cell counter. The number of cells in cultures treated with the test oligonucleotide (expressed as 100%) is compared with the number of cells in cultures treated with the control oligonucleotide. The number of cells in cultures treated with the test oligonucleotide is not more than 30% of control, indicating that the inhibition of 30 human cation transport ATPase-like protein has an anti-proliferative effect on cancer cells.

EXAMPLE 8*In vivo testing of compounds/target validation for cancer treatment*5 **Acute Mechanistic Assays***Reduction in Mitogenic Plasma Hormone Levels*

This non-tumor assay measures the ability of a compound to reduce either the endogenous level of a circulating hormone or the level of hormone produced in 10 response to a biologic stimulus. Rodents are administered test compound (p.o., i.p., i.v., i.m., or s.c.). At a predetermined time after administration of test compound, blood plasma is collected. Plasma is assayed for levels of the hormone of interest. If the normal circulating levels of the hormone are too low and/or variable to provide consistent results, the level of the hormone may be elevated by a pre-treatment with a 15 biologic stimulus (i.e., LHRH may be injected i.m. into mice at a dosage of 30 ng/mouse to induce a burst of testosterone synthesis). The timing of plasma collection would be adjusted to coincide with the peak of the induced hormone response. Compound effects are compared to a vehicle-treated control group. An F-test is preformed to determine if the variance is equal or unequal followed by a 20 Student's t-test. Significance is p value \leq 0.05 compared to the vehicle control group.

Hollow Fiber Mechanism of Action Assay

25 Hollow fibers are prepared with desired cell line(s) and implanted intraperitoneally and/or subcutaneously in rodents. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Fibers are harvested in accordance with specific readout assay protocol, these may include assays for gene expression (bDNA, PCR, or Taqman), or a specific biochemical activity (i.e., cAMP levels. Results are analyzed by Student's t-test or 30 Rank Sum test after the variance between groups is compared by an F-test, with significance at p \leq 0.05 as compared to the vehicle control group.

Subacute Functional *In Vivo* Assays***Reduction in Mass of Hormone Dependent Tissues***

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This is another non-tumor assay that measures the ability of a compound to reduce the mass of a hormone dependent tissue (i.e., seminal vesicles in males and uteri in females). Rodents are administered test compound (p.o., i.p., i.v., i.m., or s.c.) according to a predetermined schedule and for a predetermined duration (i.e., 1 week). At termination of the study, animals are weighed, the target organ is excised, any fluid is expressed, and the weight of the organ is recorded. Blood plasma may also be collected. Plasma may be assayed for levels of a hormone of interest or for levels of test agent. Organ weights may be directly compared or they may be normalized for the body weight of the animal. Compound effects are compared to a vehicle-treated control group. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test. Significance is p value ≤ 0.05 compared to the vehicle control group.

Hollow Fiber Proliferation Assay

20

Hollow fibers are prepared with desired cell line(s) and implanted intraperitoneally and/or subcutaneously in rodents. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Fibers are harvested in accordance with specific readout assay protocol. Cell proliferation is determined by measuring a marker of cell number (i.e., MTT or LDH). The cell number and change in cell number from the starting inoculum are analyzed by Student's t-test or Rank Sum test after the variance between groups is compared by an F-test, with significance at $p \leq 0.05$ as compared to the vehicle control group.

*Anti-angiogenesis Models**Corneal Angiogenesis*

Hydron pellets with or without growth factors or cells are implanted into a 5 micropocket surgically created in the rodent cornea. Compound administration may be systemic or local (compound mixed with growth factors in the hydron pellet). Corneas are harvested at 7 days post implantation immediately following intracardiac infusion of colloidal carbon and are fixed in 10% formalin. Readout is qualitative scoring and/or image analysis. Qualitative scores are compared by Rank Sum test. 10 Image analysis data is evaluated by measuring the area of neovascularization (in pixels) and group averages are compared by Student's t-test (2 tail). Significance is $p \leq 0.05$ as compared to the growth factor or cells only group.

Matrigel Angiogenesis

15 Matrigel, containing cells or growth factors, is injected subcutaneously. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Matrigel plugs are harvested at predetermined time point(s) and prepared for readout. Readout is an ELISA-based assay for hemoglobin concentration and/or histological examination (i.e. vessel count, special staining for endothelial surface markers: CD31, factor-8). Readouts are analyzed by Student's t-test, after the variance between groups is compared by an 20 F-test, with significance determined at $p \leq 0.05$ as compared to the vehicle control group.

25 Primary Antitumor Efficacy

*Early Therapy Models**Subcutaneous Tumor*

30 Tumor cells or fragments are implanted subcutaneously on Day 0. Vehicle and/or compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined

schedule starting at a time, usually on Day 1, prior to the ability to measure the tumor burden. Body weights and tumor measurements are recorded 2-3 times weekly. Mean net body and tumor weights are calculated for each data collection day. Anti-tumor efficacy may be initially determined by comparing the size of treated (T) and control (C) tumors on a given day by a Student's t-test, after the variance between groups is compared by an F-test, with significance determined at $p \leq 0.05$. The experiment may also be continued past the end of dosing in which case tumor measurements would continue to be recorded to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is $p \leq 0.05$.

15 *Intraperitoneal/Intracranial Tumor Models*

Tumor cells are injected intraperitoneally or intracranially on Day 0. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule starting on Day 1. Observations of morbidity and/or mortality are recorded twice daily. Body weights are measured and recorded twice weekly. Morbidity/mortality data is expressed in terms of the median time of survival and the number of long-term survivors is indicated separately. Survival times are used to generate Kaplan-Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment.

25

Established Disease Model

Tumor cells or fragments are implanted subcutaneously and grown to the desired size for treatment to begin. Once at the predetermined size range, mice are randomized into treatment groups. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Tumor and body weights are measured and

recorded 2-3 times weekly. Mean tumor weights of all groups over days post inoculation are graphed for comparison. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. Tumor measurements may be recorded after dosing has stopped to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is p value ≤ 0.05 compared to the vehicle control group.

Orthotopic Disease Models

15 *Mammary Fat Pad Assay*

Tumor cells or fragments, of mammary adenocarcinoma origin, are implanted directly into a surgically exposed and reflected mammary fat pad in rodents. The fat pad is placed back in its original position and the surgical site is closed. Hormones may also be administered to the rodents to support the growth of the tumors. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Tumor and body weights are measured and recorded 2-3 times weekly. Mean tumor weights of all groups over days post inoculation are graphed for comparison. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group.

30 Tumor measurements may be recorded after dosing has stopped to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the

median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is p value ≤ 0.05 compared to the vehicle control group. In addition, this model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ, or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

10

Intraprostatic Assay

Tumor cells or fragments, of prostatic adenocarcinoma origin, are implanted directly into a surgically exposed dorsal lobe of the prostate in rodents. The prostate is externalized through an abdominal incision so that the tumor can be implanted specifically in the dorsal lobe while verifying that the implant does not enter the seminal vesicles. The successfully inoculated prostate is replaced in the abdomen and the incisions through the abdomen and skin are closed. Hormones may also be administered to the rodents to support the growth of the tumors. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the animal is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., the lungs), or measuring the target organ weight (i.e., the regional lymph nodes). The means of these endpoints are compared by Student's t-test after conducting an F-test,

with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

Intrabronchial Assay

5

Tumor cells of pulmonary origin may be implanted intrabronchially by making an incision through the skin and exposing the trachea. The trachea is pierced with the beveled end of a 25 gauge needle and the tumor cells are inoculated into the main bronchus using a flat-ended 27 gauge needle with a 90° bend. Compounds are 10 administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the animal is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is preformed to determine if the variance is 15 equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., 20 the contralateral lung), or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

Intracecal Assay

25

Tumor cells of gastrointestinal origin may be implanted intracecally by making an abdominal incision through the skin and externalizing the intestine. Tumor cells are inoculated into the cecal wall without penetrating the lumen of the intestine using a 27 or 30 gauge needle. Compounds are administered p.o., i.p., i.v., i.m., or s.c. 30 according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the animal

is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment.

5 Significance is $p \leq 0.05$ as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., the liver), or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

Secondary (Metastatic) Antitumor Efficacy

15 *Spontaneous Metastasis*

Tumor cells are inoculated s.c. and the tumors allowed to grow to a predetermined range for spontaneous metastasis studies to the lung or liver. These primary tumors are then excised. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule which may include the period leading up to the excision of the primary tumor to evaluate therapies directed at inhibiting the early stages of tumor metastasis. Observations of morbidity and/or mortality are recorded daily. Body weights are measured and recorded twice weekly. Potential endpoints include survival time, numbers of visible foci per target organ, or target organ weight. When survival time is used as the endpoint the other values are not determined. Survival data is used to generate Kaplan-Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment. The mean number of visible tumor foci, as determined under a dissecting microscope, and the mean target organ weights are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment for both of these endpoints.

Forced Metastasis

Tumor cells are injected into the tail vein, portal vein, or the left ventricle of the heart
5 in experimental (forced) lung, liver, and bone metastasis studies, respectively. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Observations of morbidity and/or mortality are recorded daily. Body weights are measured and recorded twice weekly. Potential endpoints include survival time, numbers of visible foci per target organ, or target organ weight. When
10 survival time is used as the endpoint the other values are not determined. Survival data is used to generate Kaplan-Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment. The mean number of visible tumor foci, as determined under a dissecting microscope, and the mean target organ weights are compared by Student's t-test after conducting an F-test, with significance
15 at $p \leq 0.05$ compared to the vehicle control group in the experiment for both endpoints.

EXAMPLE 9

20 *In vivo testing of compounds/target validation*

Pain

Acute pain. Acute pain is measured on a hot plate mainly in rats. Two variants of hot
25 plate testing are used: In the classical variant animals are put on a hot surface (52 to 56°C) and the latency time is measured until the animals show nocifensive behavior, such as stepping or foot licking. The other variant is an increasing temperature hot plate where the experimental animals are put on a surface of neutral temperature. Subsequently this surface is slowly but constantly heated until the animals begin to
30 lick a hind paw. The temperature which is reached when hind paw licking begins is a measure for pain threshold.

Compounds are tested against a vehicle treated control group. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

5

Persistent pain. Persistent pain is measured with the formalin or capsaicin test, mainly in rats. A solution of 1 to 5% formalin or 10 to 100 µg capsaicin is injected into one hind paw of the experimental animal. After formalin or capsaicin application the animals show nocifensive reactions like flinching, licking and biting of the affected paw. The number of nocifensive reactions within a time frame of up to 90 minutes is a measure for intensity of pain.

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Compounds are tested against a vehicle treated control group. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to formalin or capsaicin administration.

15

Neuropathic pain. Neuropathic pain is induced by different variants of unilateral sciatic nerve injury mainly in rats. The operation is performed under anesthesia. The first variant of sciatic nerve injury is produced by placing loosely constrictive ligatures around the common sciatic nerve. The second variant is the tight ligation of about the half of the diameter of the common sciatic nerve. In the next variant, a group of models is used in which tight ligations or transections are made of either the L5 and L6 spinal nerves, or the L7 spinal nerve only. The fourth variant involves an axotomy of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact whereas the last variant comprises the axotomy of only the tibial branch leaving the sural and common nerves uninjured. Control animals are treated with a sham operation.

20

Postoperatively, the nerve injured animals develop a chronic mechanical allodynia, cold allodynia, as well as a thermal hyperalgesia. Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer, IITC

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Inc.-Life Science Instruments, Woodland Hills, SA, USA; Electronic von Frey System, Somedic Sales AB, Hörby, Sweden). Thermal hyperalgesia is measured by means of a radiant heat source (Plantar Test, Ugo Basile, Comerio, Italy), or by means of a cold plate of 5 to 10°C where the nocifensive reactions of the affected 5 hind paw are counted as a measure of pain intensity. A further test for cold induced pain is the counting of nocifensive reactions, or duration of nocifensive responses after plantar administration of acetone to the affected hind limb. Chronic pain in general is assessed by registering the circadian rhythms in activity (Surjo and 10 Arndt, Universität zu Köln, Cologne, Germany), and by scoring differences in gait (foot print patterns; FOOTPRINTS program, Klapdor et al., 1997. A low cost method 15 to analyze footprint patterns. J. Neurosci. Methods 75, 49-54).

Compounds are tested against sham operated and vehicle treated control groups. Substance application is performed at different time points via different application 15 routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

Inflammatory Pain. Inflammatory pain is induced mainly in rats by injection of 0.75 mg carrageenan or complete Freund's adjuvant into one hind paw. The animals develop an edema with mechanical allodynia as well as thermal hyperalgesia. 20 Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA). Thermal hyperalgesia is measured by means of a radiant heat source (Plantar Test, Ugo Basile, Comerio, Italy, Paw thermal stimulator, G. Ozaki, University of California, USA). For edema measurement two methods are being used. In the first 25 method, the animals are sacrificed and the affected hindpaws sectioned and weighed. The second method comprises differences in paw volume by measuring water displacement in a plethysmometer (Ugo Basile, Comerio, Italy).

Compounds are tested against uninflamed as well as vehicle treated control groups. 30 Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

5 *Diabetic neuropathic pain.* Rats treated with a single intraperitoneal injection of 50 to 80 mg/kg streptozotocin develop a profound hyperglycemia and mechanical allodynia within 1 to 3 weeks. Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer; IITC Inc.-Life Science Instruments, Woodland Hills, CA, USA).

10 Compounds are tested against diabetic and non-diabetic vehicle treated control groups. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

Parkinson's disease

15 *6-Hydroxydopamine (6-OH-DA) Lesion.* Degeneration of the dopaminergic nigrostriatal and striatopallidal pathways is the central pathological event in Parkinson's disease. This disorder has been mimicked experimentally in rats using single/sequential unilateral stereotaxic injections of 6-OH-DA into the medium forebrain bundle (MFB).

20 Male Wistar rats (Harlan Winkelmann, Germany), weighing 200±250 g at the beginning of the experiment, are used. The rats are maintained in a temperature- and humidity-controlled environment under a 12 h light/dark cycle with free access to food and water when not in experimental sessions. The following *in vivo* protocols are approved by the governmental authorities: All efforts are made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques.

25 30 Animals are administered pargyline on the day of surgery (Sigma, St. Louis, MO, USA; 50 mg/kg i.p.) in order to inhibit metabolism of 6-OHDA by monoamine oxidase and desmethylimipramine HCl (Sigma; 25 mg/kg i.p.) in order to prevent

uptake of 6-OHDA by noradrenergic terminals. Thirty minutes later the rats are anesthetized with sodium pentobarbital (50 mg/kg) and placed in a stereotaxic frame. In order to lesion the DA nigrostriatal pathway 4 μ l of 0.01% ascorbic acid-saline containing 8 μ g of 6-OHDA HBr (Sigma) are injected into the left medial fore-brain bundle at a rate of 1 μ l/min (2.4 mm anterior, 1.49 mm lateral, -2.7 mm ventral to Bregma and the skull surface). The needle is left in place an additional 5 min to allow diffusion to occur.

10 *Stepping Test.* Forelimb akinesia is assessed three weeks following lesion placement using a modified stepping test protocol. In brief, the animals are held by the experimenter with one hand fixing the hindlimbs and slightly raising the hind part above the surface. One paw is touching the table, and is then moved slowly sideways (5 s for 1 m), first in the forehand and then in the backhand direction. The number of adjusting steps is counted for both paws in the backhand and forehand direction of movement. The sequence of testing is right paw forehand and backhand adjusting stepping, followed by left paw forehand and backhand directions. The test is repeated three times on three consecutive days, after an initial training period of three days prior to the first testing. Forehand adjusted stepping reveals no consistent differences between lesioned and healthy control animals. Analysis is therefore restricted to 15 backhand adjusted stepping.

20 *Balance Test.* Balance adjustments following postural challenge are also measured during the stepping test sessions. The rats are held in the same position as described in the stepping test and, instead of being moved sideways, tilted by the experimenter towards the side of the paw touching the table. This maneuver results in loss of balance and the ability of the rats to regain balance by forelimb movements is scored on a scale ranging from 0 to 3. Score 0 is given for a normal forelimb placement. When the forelimb movement is delayed but recovery of postural balance detected, 25 score 1 is given. Score 2 represents a clear, yet insufficient, forelimb reaction, as evidenced by muscle contraction, but lack of success in recovering balance, and score 30 3 is given for no reaction of movement. The test is repeated three times a day on each

side for three consecutive days after an initial training period of three days prior to the first testing.

5 *Staircase Test (Paw Reaching).* A modified version of the staircase test is used for evaluation of paw reaching behavior three weeks following primary and secondary lesion placement. Plexiglass test boxes with a central platform and a removable staircase on each side are used. The apparatus is designed such that only the paw on the same side at each staircase can be used, thus providing a measure of independent forelimb use. For each test the animals are left in the test boxes for 15 min. The 10 double staircase is filled with 7 x 3 chow pellets (Precision food pellets, formula: P, purified rodent diet, size 45 mg; Sandown Scientific) on each side. After each test the number of pellets eaten (successfully retrieved pellets) and the number of pellets taken (touched but dropped) for each paw and the success rate (pellets eaten/pellets taken) are counted separately. After three days of food deprivation (12 g per animal 15 per day) the animals are tested for 11 days. Full analysis is conducted only for the last five days.

20 *MPTP treatment.* The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes degeneration of mesencephalic dopaminergic (DAergic) neurons in rodents, non-human primates, and humans and, in so doing, reproduces many of the symptoms of Parkinson's disease. MPTP leads to a marked decrease in the levels of dopamine and its metabolites, and in the number of dopaminergic terminals in the striatum as well as severe loss of the tyrosine hydroxylase (TH)-immunoreactive cell bodies in the substantia nigra, pars compacta.

25 In order to obtain severe and long-lasting lesions, and to reduce mortality, animals receive single injections of MPTP, and are then tested for severity of lesion 7-10 days later. Successive MPTP injections are administered on days 1, 2 and 3. Animals receive application of 4 mg/kg MPTP hydrochloride (Sigma) in saline once daily. All 30 injections are intraperitoneal (i.p.) and the MPTP stock solution is frozen between injections. Animals are decapitated on day 11.

Immunohistology. At the completion of behavioral experiments, all animals are anaesthetized with 3 ml thiopental (1 g/40 ml i.p., Tyrol Pharma). The mice are perfused transcardially with 0.01 M PBS (pH 7.4) for 2 min, followed by 4% paraformaldehyde (Merck) in PBS for 15 min. The brains are removed and placed in 4% paraformaldehyde for 24 h at 4°C. For dehydration they are then transferred to a 20% sucrose (Merck) solution in 0.1 M PBS at 4°C until they sink. The brains are frozen in methylbutan at -20°C for 2 min and stored at -70°C. Using a sledge microtome (mod. 3800-Frigocut, Leica), 25 µm sections are taken from the genu of the corpus callosum (AP 1.7 mm) to the hippocampus (AP 21.8 mm) and from AP 24.16 to AP 26.72. Forty-six sections are cut and stored in assorters in 0.25 M Tris buffer (pH 7.4) for immunohistochemistry.

15 A series of sections is processed for free-floating tyrosine hydroxylase (TH) immunohistochemistry. Following three rinses in 0.1 M PBS, endogenous peroxidase activity is quenched for 10 min in 0.3% H₂O₂ ± PBS. After rinsing in PBS, sections are preincubated in 10% normal bovine serum (Sigma) for 5 min as blocking agent and transferred to either primary anti-rat TH rabbit antiserum (dilution 1:2000).

20 Following overnight incubation at room temperature, sections for TH immunoreactivity are rinsed in PBS (2 x 10 min) and incubated in biotinylated anti-rabbit immunoglobulin G raised in goat (dilution 1:200) (Vector) for 90 min, rinsed repeatedly and transferred to Vectastain ABC (Vector) solution for 1 h. 3,3'-25 -Diaminobenzidine tetrahydrochloride (DAB; Sigma) in 0.1 M PBS, supplemented with 0.005% H₂O₂, serves as chromogen in the subsequent visualization reaction. Sections are mounted on to gelatin-coated slides, left to dry overnight, counterstained with hematoxylin dehydrated in ascending alcohol concentrations and cleared in butylacetate. Coverslips are mounted on entellan.

30.

5 *Rotarod Test.* We use a modification of the procedure described by Rozas and Labandeira-Garcia (1997), with a CR-1 Rotamex system (Columbus Instruments, Columbus, OH) comprising an IBM-compatible personal computer, a CIO-24 data acquisition card, a control unit, and a four-lane rotarod unit. The rotarod unit consists of a rotating spindle (diameter 7.3 cm) and individual compartments for each mouse.

10 The system software allows preprogramming of session protocols with varying rotational speeds (0–80 rpm). Infrared beams are used to detect when a mouse has fallen onto the base grid beneath the rotarod. The system logs the fall as the end of the experiment for that mouse, and the total time on the rotarod, as well as the time of the fall and all the set-up parameters, are recorded. The system also allows a weak current to be passed through the base grid, to aid training.

Dementia

15 *The object recognition task.* The object recognition task has been designed to assess the effects of experimental manipulations on the cognitive performance of rodents. A rat is placed in an open field, in which two identical objects are present. The rats inspects both objects during the first trial of the object recognition task. In a second trial, after a retention interval of for example 24 hours, one of the two objects used in

20 the first trial, the 'familiar' object, and a novel object are placed in the open field. The inspection time at each of the objects is registered. The basic measures in the OR task is the time spent by a rat exploring the two object the second trial. Good retention is reflected by higher exploration times towards the novel than the 'familiar' object.

25 Administration of the putative cognition enhancer prior to the first trial predominantly allows assessment of the effects on acquisition, and eventually on consolidation processes. Administration of the testing compound after the first trial allows to assess the effects on consolidation processes, whereas administration before the second trial allows to measure effects on retrieval processes.

The passive avoidance task. The passive avoidance task assesses memory performance in rats and mice. The inhibitory avoidance apparatus consists of a two-compartment box with a light compartment and a dark compartment. The two compartments are separated by a guillotine door that can be operated by the experimenter. A threshold of 2 cm separates the two compartments when the guillotine door is raised. When the door is open, the illumination in the dark compartment is about 2 lux. The light intensity is about 500 lux at the center of the floor of the light compartment.

10 Two habituation sessions, one shock session, and a retention session are given, separated by inter-session intervals of 24 hours. In the habituation sessions and the retention session the rat is allowed to explore the apparatus for 300 sec. The rat is placed in the light compartment, facing the wall opposite to the guillotine door. After an accommodation period of 15 sec. the guillotine door is opened so that all parts of 15 the apparatus can be visited freely. Rats normally avoid brightly lit areas and will enter the dark compartment within a few seconds.

20 In the shock session the guillotine door between the compartments is lowered as soon as the rat has entered the dark compartment with its four paws, and a scrambled 1 mA footshock is administered for 2 sec. The rat is removed from the apparatus and put back into its home cage. The procedure during the retention session is identical to that of the habituation sessions.

25 The step-through latency, that is the first latency of entering the dark compartment (in sec.) during the retention session is an index of the memory performance of the animal; the longer the latency to enter the dark compartment, the better the retention is. A testing compound is given half an hour before the shock session, together with 30 1 mg*kg⁻¹ scopolamine. Scopolamine impairs the memory performance during the retention session 24 hours later. If the test compound increases the enter latency compared with the scopolamine-treated controls, is likely to possess cognition enhancing potential.

5 *The Morris water escape task.* The Morris water escape task measures spatial orientation learning in rodents. It is a test system that has extensively been used to investigate the effects of putative therapeutic on the cognitive functions of rats and mice. The performance of an animal is assessed in a circular water tank with an escape platform that is submerged about 1 cm below the surface of the water. The escape platform is not visible for an animal swimming in the water tank. Abundant extra-maze cues are provided by the furniture in the room, including desks, computer equipment, a second water tank, the presence of the experimenter, and by a radio on a 10 shelf that is playing softly.

15 The animals receive four trials during five daily acquisition sessions. A trial is started by placing an animal into the pool, facing the wall of the tank. Each of four starting positions in the quadrants north, east, south, and west is used once in a series of four trials; their order is randomized. The escape platform is always in the same position. A trial is terminated as soon as the animal had climbs onto the escape platform or when 90 seconds have elapsed, whichever event occurs first. The animal is allowed to stay on the platform for 30 seconds. Then it is taken from the platform and the next trial is started. If an animal did not find the platform within 90 seconds it is put 20 on the platform by the experimenter and is allowed to stay there for 30 seconds. After the fourth trial of the fifth daily session, an additional trial is given as a probe trial: the platform is removed, and the time the animal spends in the four quadrants is measured for 30 or 60 seconds. In the probe trial, all animals start from the same start position, opposite to the quadrant where the escape platform had been positioned 25 during acquisition.

30 Four different measures are taken to evaluate the performance of an animal during acquisition training: escape latency, traveled distance, distance to platform, and swimming speed. The following measures are evaluated for the probe trial: time (s) in quadrants and traveled distance (cm) in the four quadrants. The probe trial provides additional information about how well an animal learned the position of the

escape platform. If an animal spends more time and swims a longer distance in the quadrant where the platform had been positioned during the acquisition sessions than in any other quadrant, one concludes that the platform position has been learned well.

5 In order to assess the effects of putative cognition enhancing compounds, rats or mice with specific brain lesions which impair cognitive functions, or animals treated with compounds such as scopolamine or MK-801, which interfere with normal learning, or aged animals which suffer from cognitive deficits, are used.

10 *The T-maze spontaneous alternation task.* The T-maze spontaneous alternation task (TeMCAT) assesses the spatial memory performance in mice. The start arm and the two goal arms of the T-maze are provided with guillotine doors which can be operated manually by the experimenter. A mouse is put into the start arm at the beginning of training. The guillotine door is closed. In the first trial, the 'forced trial', 15 either the left or right goal arm is blocked by lowering the guillotine door. After the mouse has been released from the start arm, it will negotiate the maze, eventually enter the open goal arm, and return to the start position, where it will be confined for 5 seconds, by lowering the guillotine door. Then, the animal can choose freely between the left and right goal arm (all guillotine-doors opened) during 14 'free choice' trials. As soon as the mouse has entered one goal arm, the other one is closed. 20 The mouse eventually returns to the start arm and is free to visit whichever go alarm it wants after having been confined to the start arm for 5 seconds. After completion of 14 free choice trials in one session, the animal is removed from the maze. During training, the animal is never handled.

25 The percent alternations out of 14 trials is calculated. This percentage and the total time needed to complete the first forced trial and the subsequent 14 free choice trials (in s) is analyzed. Cognitive deficits are usually induced by an injection of scopolamine, 30 min before the start of the training session. Scopolamine reduced the 30 per-cent alternations to chance level, or below. A cognition enhancer, which is

always administered before the training session, will at least partially, antagonize the scopolamine-induced reduction in the spontaneous alternation rate.

EXAMPLE 10

5

In vivo testing of compounds/target validation for the treatment of genito-urinary diseases:

Bladder outlet obstruction model

10

Wistar rats (200~250 g / Charles River Japan) are anesthetized intraperitoneally with ketamine. The abdomen is opened through a midline incision and the bladder and the proximal urethra are exposed. A constant degree of urethral obstruction is produced by tying a ligature around the urethra and a catheter with an outer diameter of 1 mm.

15

The abdominal wall is closed and the animals allowed to recover.

20

After 6 weeks, the rats are anesthetized with ketamine, and the ligature around the urethra is carefully removed to normalize the outlet resistance and enable repetitive micturition. A polyethylene catheter is implanted in the bladder through the dome, and exteriorized at the scapular level. Animals are then allowed to recover for at least 48 hours.

25

Cytometric investigation is performed without anesthesia two days after bladder catheter implantation in control and obstructed animals. The bladder catheter was connected via a T-tube to a strain gauge and a microinjection pump. The conscious rats are held under partial restraint in a restraining device. Warmed saline is infused into the bladder at a rate of 3 ml/hr for control and obstructed animals. The rate of infusion is increased from 3 to 10 ml/hr to obtain similar interval times between micturitions in obstructed and control rats. Overactivity of the obstructed bladders is assessed by measuring the cystometric parameters such as basal pressure, peak micturition pressure, threshold pressure, micturition interval, amplitude and

30

frequency of spontaneous activity and micturition slope. Lhuu *et al.*, *J. Urol.* 160, 2253-57, 1998.

5 A test compound is dissolved in an appropriate vehicle, such as a mixture of ethanol, Tween 80 (ICN Biomedicals Inc.), and saline (1:1:8, v/v/v), is administered intravenously through the catheter:

EXAMPLE 11

10 *In vivo testing of compounds/target validation for the treatment of genito-urinary diseases*

Measurement of the relaxation effects on the rat prostate contraction

Organ bath assay for measuring agonist-induced contraction of prostate

15 An organ bath assay is employed to measure the agonist-induced contraction of prostate for assessing the biological activity of test compounds (*i.e.*, drug candidates). Male Wistar rats (200~250 g / Charles River Japan) are anesthetized with ether and sacrificed by dislocating the necks. The whole prostate is excised and placed in oxygenated Modified Krebs-Henseleit solution (pH 7.4) of the following composition (112 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 2 mM CaCl₂, 2.5 mM NaHCO₃, 12 mM glucose). Ventricle prostate lobes were dissected into several strips depending on the size of prostate. Prostate strips are equilibrated for 60 min in organ bath chambers before any stimulation.

25 Isometric tension is recorded under an appropriate load. Contractile response to adrenergic agonists or electric field stimulation is determined several times until reproducible responses are obtained. Test compounds are pre-incubated prior to the agonistic or electric stimulation. The ratio of each contraction to the negative control is calculated and the effect of the test compounds on the prostate contraction is evaluated.

EXAMPLE 12

5 In vivo testing of compounds/target validation for the treatment of genito-urinary diseases

Evaluation of test compounds for micturition disorders

Micturition parameters from cystometry are utilized to evaluate the drug candidates for micturition disorders. Sprague-Dawley rats are anesthetized by intraperitoneal administration of urethane at 1.2 g/kg. The abdomen is opened through a midline incision, and a polyethylene catheter is implanted into the bladder through the dome. In parallel, the inguinal region is incised, and a polyethylene catheter filled with 2 IU/ml of heparin in saline is inserted into a common iliac artery. The bladder catheter is connected via T-tube to a pressure transducer and a microinjection pump. Saline is infused at room temperature into the bladder at a rate of 2.4 ml/hr. Intravesicular pressure is recorded continuously on a chart pen recorder. At least three reproducible micturition cycles are recorded before a test compound administration and used as baseline values. The saline infusion is stopped before administrating compounds. A test compound dissolved in an appropriate vehicle is intraarterially injected 2 min before another intraarterial administration of stimulant such as capsaicin. Relative increases in the induced intravesicular pressure are analyzed from the cystometry data in comparison with the normal micturition patterns. The test compounds-mediated inhibition of the increased bladder pressures is evaluated using Student's t-test. A probability level less than 5% is accepted as significant difference.

EXAMPLE 13**In vivo testing of compounds/target validation for the treatment of genito-urinary diseases**

5

Measurement of the relaxation effects on the rat bladder contraction**Organ bath assay for measuring agonist-induced contraction of urinary bladder**

An organ bath assay is employed to measure the agonist-induced contraction of urinary bladder for assessing the biological activity of test compounds (i.e., drug candidates). Male Wistar rats (200~250 g / Charles River Japan) are anesthetized with ether and sacrificed by dislocating the necks. The whole urinary bladder is excised and placed in oxygenated Modified Krebs-Henseleit solution (pH 7.4) of the following composition (112 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 2 mM CaCl₂, 2.5 mM NaHCO₃, 12 mM glucose).

Isometric tension is recorded under an appropriate load using longitudinal strips of rat detrusor muscle. Bladder strips are equilibrated for 60 minutes before each stimulation. Contractile response to 80 mM KCl is determined at 15 minute intervals until reproducible responses are obtained. The response to KCl is used as an internal standard to evaluate the effect of test compounds.

The effects of test compounds are investigated by incubating the strips with compounds for 30 minutes prior to stimulation with an appropriate agonist or electrical stimulation. One of the preparations made from the same animal serves as a control, while others are used for evaluating test compounds. The ratio of each contraction to the internal standard (e.g., a KCl-induced contraction) is calculated, and the effects of the test compounds on the contraction are evaluated.

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EXAMPLE 14

In vivo testing of compounds/target validation for the treatment of genito-urinary diseases

5

Measurement of bladder cystometry in anesthetized rats

(1) Animals. Female Sprague-Dawley rats (200~250 g / Charles River Japan) are used.

10

(2) Catheter implantation. Rats are anesthetized by intraperitoneal administration of urethane (Sigma) at 1.25 g/kg. The abdomen is opened through a midline incision, and a polyethylene catheter (BECTON DICKINSON, PE50) is implanted into the bladder through the dome. In parallel, the inguinal region is incised, and a polyethylene catheter (BECTON DICKINSON, PE50) filled with saline (Otsuka) is inserted into a femoral vein.

15

(3) Investigation of bladder contraction. The bladder is filled via the catheter by incremental volume of saline until spontaneous bladder contractions occur. The intravesicular pressure is measured a pressure transducer and displayed continuously on a chart recorder. The activity of test compounds is assessed after intravenous administration through a polyethylene cannula inserted into the femoral vein.

20

Measurement of bladder cystometry in conscious rats

(1) Animals. Female Sprague-Dawley rats (200~250 g / Charles River Japan) are used.

30

(2) Catheter implantation. Rats are anesthetized by intramuscular administration of ketamine (75 mg/kg) and xylazine (15 mg/kg). The abdomen is opened

5 through a midline incision, and a polyethylene catheter (BECTON DICKINSON, PE50) is implanted into the bladder through the dome. The catheter is tunneled through subcutis of the animal by needle (14G) to neck. In parallel, the inguinal region is incised, and a polyethylene catheter (BECTON DICKINSON, PE50) filled with saline (Otsuka) is inserted into a femoral vein. The catheter is tunneled through subcutis of the animal by needle to neck.

10 (3) Cystometric investigation. The bladder catheter is connected via T-tube to a pressure transducer (Viggo-Spectramed Pte Ltd, DT-XXAD) and a micro-injection pump (TERUMO). Saline is infused at room temperature into the bladder at a rate of 10 ml/hr. Intravesicular pressure is recorded continuously on a chart pen recorder (Yokogawa). At least three reproducible micturition cycles are recorded before a test compound administration.

15 (4) Administration of test compounds. A test compound dissolved in the mixture of ethanol, Tween 80 (ICN Biomedicals Inc.) and saline (1 : 1 : 8, v/v/v) is administered intravenously through the catheter.

20 **EXAMPLE 15**

In vitro testing of compounds/target validation for hematological diseases
Isolation of CD34⁺ cells

25 Mononuclear cells from fresh blood (cord blood, peripheral blood, bone marrow) were separated by Ficoll Paque[®] (1.077 density, Amersham-Pharmacia) density gradient centrifugation, and CD34⁺ cells were purified by immunomagnetic separation system (MiniMACS, Miltenyi Biotec), according to the manufacturer's instructions (Direct CD34 Progenitor Cell Isolation Kit, Miltenyi Biotec). The 30 percentage of CD34⁺ cells were generally from 90-95%.

*Erythropoiesis/Anemia**Erythroid CD34⁺ Liquid Culture*

1-2 x 10⁴ CD34⁺ cells were plated in triplicate in 24-well plates with 1ml Iscoves
5 modified Dulbecco medium (IMDM) (Invitrogen) containing 10% fetal bovine serum
(FCS, Invitrogen), 1% Glutamine (Invitrogen) supplemented with SCF (25 ng/ml)
(PeproTech), different concentration of Erythropoietin (0.01 U/ml – 1 U/ml) (Erypo[®]
FS 4000, Cilag) with or without compounds. Control cells were incubated with 0.1-
0.2% DMSO instead of compounds. The cultures were incubated at 37°C in a fully
10 humidified atmosphere with 5% CO₂. After 9 to 14 days cells were harvested,
counted and stained with phycoerythrin (PE)-conjugated mAb against Glycophorin A
15 (Pharmingen) to analyze differentiation.

Erythroid Colony-forming assay

15 Five hundred CD34⁺ cells/ml were plated in triplicate 24-well plates with 1%
methylcellulose in IMDM containing 30% FCS, 1% bovine serum albumin (BSA),
2 mM L-glutamine and 10-4 M 2-mercaptoethanol (Methocult H4230, Cell
Systems[®]), IL-3 (10 ng/ml) (PeproTech) with different concentration of erythro-
20 poietin (0.01 U/ml – 1 U/ml) with or without compounds. The cultures were
incubated at 37°C in a fully humidified atmosphere with 5% CO₂. After 9 to 14 days
erythroid burst forming units (BFU-E) were counted from each of the plates.
Afterwards cells were dissolved from methylcellulose with 0.1% NaCl solution.
Cells were counted and stained with phycoerythrin (PE)-conjugated mAb against
25 Glycophorin A (Pharmingen) to analyze differentiation.

BFU-E culture

1 x 10⁵ Cord Blood CD34⁺ cells/ml were cultured in IMDM containing 15% BIT-
30 9500 (Cell Systems[®]), supplemented with IL-3 (10 ng/ml), IL-6 (10 ng/ml) and SCF
(25ng/ml) (PeproTech) and incubated at 37°C in a fully humidified atmosphere with

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5% CO₂. 3 and 5 days after initiation of culture an equal volume of fresh medium supplemented with 2 X cytokines were added. On day 6 to 7 1-2x10⁴ erythroid progenitors were plated in triplicate in 24-well plates with 1 ml IMDM containing 10% FCS, 1% glutamine supplemented with SCF (25 ng/ml), different concentration of erythropoietin (0.01 U/ml – 1 U/ml) with or without compounds. Control cells were incubated with 0.1-0.2% DMSO instead of compounds. The cultures were incubated at 37°C in a fully humidified atmosphere with 5% CO₂. After 6 to 8 days cells were harvested and counted to analyze proliferation.

10 *CD36⁺ cells*

1 x 10⁵ Cord Blood CD34⁺ cells/ml were cultured in IMDM containing 15% BIT-9500 supplemented with IL-3 (10 ng/ml), IL-6 (10 ng/ml) and SCF (25 ng/ml) and incubated at 37°C in a fully humidified atmosphere with 5% CO₂. 3 and 5 days after initiation of culture an equal volume of fresh medium supplemented with 2 X cytokines were added. On day 6 to 7 cells were stained with PE-conjugated mAb against CD36 (Pharmingen) and CD36⁺ cells were purified using anti-PE microbeads and Mini MACS system (Miltenyi Biotec) according to the manufacturer's instructions. 1-2x10⁴ CD36⁺ cells were plated in triplicate 24well plates with 1ml IMDM containing 10% FCS, 1% Glutamine supplemented with SCF (25 ng/ml), different concentration of Erythropoietin (0.01 U/ml – 1 U/ml) with or without compounds. Control cells were incubated with 0.1-0.2% DMSO instead of compounds. The cultures were incubated at 37°C in a fully humidified atmosphere with 5% CO₂. After 6 to 8 days cells were harvested and counted to analyze proliferation.

Myelopoiesis and Thrombocytopoiesis

Myeloid CD34⁺ Liquid Culture

30 5x 10³ CD34⁺ cells isolated from peripheral blood, cord blood or from bone marrow were pre-incubated in quadruplicate in 24-well plates in 1ml medium (StemSpan)

with 15% FCS, SCF (20 ng/ml) and GM-CSF (2,5 ng/ml) for 6 to 7 days at 37°C and 5.5% CO₂. Then compounds (0.1,1 or 10 µM in DMSO) with or without G-CSF (0.25 ng/ml; Neupogen®) were added and incubated for another 6 to 7 days. The number of the early myelopoietic CD15⁺/CD11b⁻ cells and the number of the late 5 myelopoietic CD15⁺/CD11b⁺ cells were determined by cell count (proliferation) and FACS (fluorescent associated cell sorting) analysis (differentiation) at day 13-14.

Megakaryoid CD34⁺ Liquid Culture

10 5 x 10³ CD34⁺ cells isolated from peripheral blood, cord blood or from bone marrow were incubated in quadruplicate 24-well plates in 1 ml serum-free medium with 2% BSA, SCF (20 ng/ml) and compounds (0.1,1 or 10 µM in DMSO) with or without TPO (0-10 ng/ml) for 12 to 13 days at 37°C and 5% CO₂. The number of the megakaryoid CD41⁺ cells (scatter profile) were determined by FACS analysis. 15 Megakaryocytes will be examined by microscope if necessary.

In vivo testing of compounds/target validation

Erythropoiesis/Anemia

20 Compounds which have demonstrated effects on the drug target *in vitro* have been administered to normal or anemic animals orally or parenterally. In most cases, mice were used for compound testing. In some cases, other species, *e.g.* rats, hamsters or guinea pigs have been used in addition. Usually, repeated dosage is required for detection of changes in peripheral blood parameters. During the dosage period and up 25 to five days after the last administration blood samples were drawn for analysis of red and white blood cell counts as well as platelet counts using an automated blood analyzer. In addition, erythropoiesis was assessed by manual hematocrit and reticulocyte count determination. For specific analysis of leukocyte differentiation fluorescent associated cell sorting (FACS) was used.

*Myelopoiesis and Thrombocytopoiesis**Myelopoiesis*

5 Immunocompetent Balb/c mice were treated with compounds at different doses (based on pharmacokinetic data) once/day or bid per-orally or parenterally for up to 4 days. The WBC (white blood cells count) and the neutrophil count were monitored by FACS (CD11b⁺; scatter properties).

10 Immunocompromised Balb/c were generated by intravenous treatment with 5-FU (100 mg/kg i.p.). 24 hours later the mice were treated with the test compound at different doses (based on pharmacokinetic data) once/day or bid per-orally or parenterally for up to 7 to 13 days. Peripheral blood counts (WBC, RBC, PLT) have been determined after retroorbital plexus puncture at days 5,7,11 and 14. For more detailed investigations the development of cellularity of femoral bone marrow and 15 spleen were investigated by FACS analysis. The expression of specific differentiation markers on stem and progenitor cells (e.g. CD34, CD33, CD38, CD11b) and scatter properties were investigated.

Thrombocytopoiesis

20 Thrombopoietic compounds at different doses (based on pharmacokinetic data) were administered orally or parenterally following chemotherapy (Carboplatin, 100 mg/kg i.p) immunocompromised mice. After repeated administration (once/day or bid for five to seven days) peripheral blood platelets (automated blood analyzer) have been 25 determined after retroorbital plexus puncture at day 5, 7, 11, and 14.

EXAMPLE 16*In vivo target validation for the treatment of atherosclerosis*

5 Effects on plasma cholesterol levels including HDL cholesterol are typically assessed in humanized apo-AI transgenic mice. Modulation of human target proteins can be determined in corresponding transgenic mice (e.g., CETP transgenic mice). Triglyceride lowering is usually evaluated in ob/ob mice or Zucker rats. Animals are fed with normal diets or modified diets (e.g., enriched by 0.5 % cholesterol 20% coconut oil). Standard protocols consist of oral applications once daily for 7 to 10 days at doses ranging from 0,1 to 100 mg/kg. The compounds are dissolved (e.g., in Solutol/Ethanol/saline mixtures) and applied by oral gavage or intravenous injection. Before and at the end of the application period, blood samples are typically drawn by retroorbital punctuation. Plasma cholesterol and triglyceride levels are determined 10 with standardized clinical diagnostic kits (e.g., INFINITY™ cholesterol reagent and INFINITY™ triglyceride reagent; Sigma, St. Louis). HDL cholesterol is determined 15 after phosphotungstic acid precipitation of non-HDL lipoproteins or FPLC gel filtration with post-column derivatization of cholesterol using the reagents mentioned above. Plasma levels of human apolipoprotein-AI in relevant humanized transgenic 20 mice are measured by immunoturbidimetry (Sigma).

Long-term anti-atherosclerotic potency of drug candidates are evaluated in Apo E- 25 knockout mice. Therefore, animals are fed a standard chow diet (4,5% fat) or a Western diet (20% fat) containing 1 to 100 mg/kg of the respective compounds for 3 to 5 month. Arterial lesions are quantified in serial cryosections of the proximal aorta by staining with Oil Red O and counterstaining with hematoxylin. Lesion area size is determined using an digital imaging system.

EXAMPLE 17*In vivo testing of cardiovascular effects of test compounds**Hemodynamics in anesthetized rats*

5

Male Wistar rats weighing 300-350 g (Harlan Winkelmann, Borch, Germany) are anesthetized with thiopental "Nycomed" (Nycomed, Munich, Germany) 100 mg kg⁻¹ i.p. A tracheotomy is performed, and catheters are inserted into the femoral artery for blood pressure and heart rate measurements (Gould pressure transducer and recorder, model RS 3400) and into the femoral vein for substance administration. The animals are ventilated with room air and their body temperature is controlled. Test compounds are administered orally or intravenously.

10

Hemodynamics in conscious SHR

15

Female conscious SHR (Moellegaard/Denmark, 220 – 290 g) are equipped with implantable radiotelemetry, and a data acquisition system (Data Sciences, St. Paul, MN, USA); comprising a chronically implantable transducer/transmitter unit equipped with a fluid-filled catheter is used. The transmitter is implanted into the peritoneal cavity, and the sensing catheter is inserted into the descending aorta.

20

Single administration of test compounds is performed as a solution in Transcutol®/Cremophor®/ H₂O (10/20/70 = v/v/v) given orally by gavage. The animals of control groups only receive the vehicle. Before treatment, mean blood pressure and heart rate of treated and untreated control groups are measured.

25

Hemodynamics in anesthetized dogs

30

Studies are performed on anesthetized dogs of either sex (body weight between 20-30 kg). Anesthesia is initiated by slow intravenous injection of 25 mg kg⁻¹ sodium thiopental (Trapanal®, Byk Gulden, Konstanz, Germany). The anesthesia is

continued and maintained throughout the experiment by continuous infusion of 0.04 mg kg⁻¹ h⁻¹ fentanyl (Fentanyl[®], Janssen, Neuss, Germany) and 0.25 mg kg⁻¹ h⁻¹ droperidol (DihydrobenzperidolR, Janssen, Neuss, Germany). During this anaesthesia, heart rate is as low as 35-40 bpm due to increased vagal tone. Therefore, 5 a parasympathetic blockade is achieved by intermittent injections of atropine (0.1 mg per animal) (AtropinsulfatR, Eifelfango, Bad Neuenahr, Germany). After intubation the animals are artificially ventilated at constant volume (EngströmR 300, Engström, Sweden) with room air enriched with 30% oxygen to maintain an end-tidal CO₂-concentration of about 5% (NormocapR, Datex, Finland).

10

The following catheters are implanted for measurement of cardiovascular parameters: a tip catheter for recording of left ventricular pressure is inserted into the ventricle via the carotid artery (PC350, Millar Instruments, Houston, TX, USA), a hollow catheter is inserted into the femoral artery and connected to a strain gauge (type 4-327-1, 15 Telos Medical, Upland, CA, USA for recording of arterial blood pressure, two venous catheters are inserted into either femoral vein and one additional catheter into a forearm vein for application of the anaesthetic and drugs, respectively, and an oxymetry catheter for recording of oxygen saturation is inserted into the coronary sinus via the jugular vein (Schwarzer IVH4, München, Germany).

20

After a left-sided thoracotomy the ramus circumflexus of the left coronary artery (LCX) is freed from connective tissue, and an electromagnetic flow probe (Gould Statham, Oxnard, CA, USA) is applied for measurement of coronary blood flow. Arterial blood pressure, electrocardiogram (lead II), left ventricular pressure, first 25 derivative of left ventricular pressure (dP/dt), heart rate, coronary blood flow, and oxygen saturation in the coronary sinus are continuously recorded on a pen recorder (Brush, Gould, Cleveland, OH, USA). The maximum of dP/dt is used as measure of left ventricular contractility (dP/dtmax). After completion of the instrumentation, an interval of 60 min is allowed for stabilization before the test compound is 30 intravenously applied as bolus injections. Care is taken that all measured cardiovascular parameters have returned to control level before injection of the next

dose. Each dose of the test compound is tested at least three times in different animals. The order of injection of the different doses is randomized in each animal.

EXAMPLE 18

5

Expression Profiling

Total cellular RNA was isolated from cells by one of two standard methods: 1) guanidine isothiocyanate/Cesium chloride density gradient centrifugation [Kellogg *et al.* (1990)]; or with the Tri-Reagent protocol according to the manufacturer's 10 specifications (Molecular Research Center, Inc., Cincinnati, Ohio). Total RNA prepared by the Tri-reagent protocol was treated with DNase I to remove genomic DNA contamination.

15 For relative quantitation of the mRNA distribution of the novel human prolyl hydroxylase, total RNA from each cell or tissue source was first reverse transcribed. Eighty-five µg of total RNA was reverse transcribed using 1 µmole random hexamer primers, 0.5 mM each of dATP, dCTP, dGTP and dTTP (Qiagen, Hilden, Germany) and 3000 U RnaseQut (Invitrogen, Groningen, Netherlands) in a final volume of 20 680 µl. The first strand synthesis buffer and Omniscript reverse transcriptase (2 u/µl) were obtained from (Qiagen, Hilden, Germany). The reaction was incubated at 37°C for 90 minutes and cooled on ice. The volume was adjusted to 6800 µl with water, yielding a final concentration of 12.5 ng/µl of starting RNA.

25 For relative quantitation of the distribution of the novel human prolyl hydroxylase mRNA in cells and tissues the Perkin Elmer ABI Prism RTM 7700 Sequence Detection system or Biorad iCycler was used according to the manufacturer's specifications and protocols. PCR reactions were set up to quantitate the novel human prolyl hydroxylase and the housekeeping genes HPRT (hypoxanthine 30 phosphoribosyltransferase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), β-actin, and others. Forward and reverse primers and probes for the novel human

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prolyl hydroxylase were designed using the Perkin Elmer ABI Primer ExpressTM software and were synthesized by TibMolBiol (Berlin, Germany). The novel human prolyl hydroxylase forward primer sequence was: Primer1
5 gcagtggagacatgtgaa (SEQ ID NO: 6). The novel human prolyl hydroxylase reverse primer sequence was Primer2 tcactggatgacagcaggac (SEQ ID NO: 7). Probe1 cgtcaatggcagtatcttccagca (SEQ ID NO: 8), labeled with FAM (carboxyfluorescein succinimidyl ester) as the reporter dye and TAMRA (carboxytetramethylrhodamine) as the quencher, was used as a probe for the novel human prolyl hydroxylase. The following reagents were prepared in a total of 25 μ l:
10 1x TaqMan buffer A, 5.5 mM MgCl₂, 200 nM of dATP, dCTP, dGTP, and dUTP, 0.025 U/ μ l AmpliTaq GoldTM, 0.01 U/ μ l AmpErase, and Probe1
cgtcaatggcagtatcttccagca (SEQ ID NO: 8), novel human prolyl hydroxylase forward and reverse primers each at 200 nM, 200 nM, novel human prolyl hydroxylase FAM/TAMRA-labeled probe, and 5 μ l of template cDNA.
15 Thermal cycling parameters were 2 min at 50°C, followed by 10 min at 95°C, followed by 40 cycles of melting at 95°C for 15 sec and annealing/extending at 60°C for 1 min.

Calculation of corrected CT values

20 The CT (threshold cycle) value is calculated as described in the "Quantitative determination of nucleic acids" section. The CF-value (factor for threshold cycle correction) is calculated as follows:
25

- 1) PCR reactions were set up to quantitate the housekeeping genes (HKG) for each cDNA sample.
- 2) CTHKG-values (threshold cycle for housekeeping gene) were calculated as described in the "Quantitative determination of nucleic acids" section.

30

3) CTHKG-mean values (CT mean value of all HKG tested on one cDNAs) of all HKG for each cDNA are calculated (n = number of HKG):

$$CTHKG\text{-}n\text{-mean value} = (CTHKG1\text{-value} + CTHKG2\text{-value} + \dots + CTHKG-n\text{-value}) / n$$

4) CTpanel mean value (CT mean value of all HKG in all tested cDNAs) = $(CTHKG1\text{-mean value} + CTHKG2\text{-mean value} + \dots + CTHKG-y\text{-mean value}) / y$ (y = number of cDNAs)

5) CFcDNA-n (correction factor for cDNA n) = CTpanel-mean value - CTHKG-n-mean value

6) CTcDNA-n (CT value of the tested gene for the cDNA n) + CFcDNA-n (correction factor for cDNA n) = CT cor-cDNA-n (corrected CT value for a gene on cDNA n)

Calculation of relative expression

20 Definition: highest CTcor-cDNA-n¹ 40 is defined as CTcor-cDNA [high]
 Relative Expression = $2(CTcor\text{-cDNA}[high] - CTcor\text{-cDNA-n})$

The following human tissues were tested: cerebellum, cerebellum (left), Alzheimer cerebral cortex, cerebellum (right), tonsilla cerebelli, postcentral gyrus, vermis cerebelli, parietal lobe, cerebral peduncles, precentral gyrus, occipital lobe, Alzheimer brain frontal lobe, hippocampus, cerebral cortex, temporal lobe, frontal lobe, retina, Alzheimer brain, pons, brain, thalamus, aorta, lung tumor, spinal cord, corpus callosum, breast, testis, artery, neuroblastoma IMR32 cells, cerebral meninges, heart ventricle (left), dorsal root ganglia, ovary tumor, thrombocytes, vein, penis, fetal kidney, rectum, lymph node, fetal brain, prostate, liver liver cirrhosis, bone marrow CD15⁺ cells, esophagus, ileum, adrenal gland, heart atrium (left),

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neuroblastoma SH5Y cells, aorta sclerotic, HEK 293 cells, small intestine, erythrocytes, breast tumor, lung COPD, cord blood CD71⁺ cells, stomach, colon tumor, heart atrium (right), fetal lung, HEP G2 cells, skeletal muscle, bone marrow CD71⁺ cells, bone marrow CD34⁺ cells, neuroblastoma SK-N-MC cells, HUVEC cells, bladder, coronary artery sclerotic, coronary artery smooth muscle primary cells, fetal aorta, skin, fetal heart, colon, prostate BPH, pericardium, substantia nigra, lung, cervix, MDA MB 231 cells (breast tumor), Jurkat (T-cells), placenta, interventricular septum, leukocytes (peripheral blood), stomach tumor, thyroid, ileum chronic inflammation, pancreas liver cirrhosis, uterus, trachea, liver tumor, adipose, thyroid tumor, heart, kidney tumor, spleen liver cirrhosis, salivary gland, coronary Artery, bone marrow, esophagus tumor, kidney, ileum tumor, liver, spleen, uterus tumor, mammary gland, fetal lung fibroblast cells, fetal liver, pancreas, thymus, HeLa cells (cervix tumor).

15 *Expression Profile*

The results of the mRNA quantification (expression profiling) are shown in Table 1.

20 **Table 1**

Tissue	Relative Expression
cerebellum	138545
cerebellum (left)	80127
Alzheimer cerebral cortex	73732
cerebellum (right)	70240
tonsilla cerebelli	70240
postcentral gyrus	50711
vermis cerebelli	44762
parietal lobe	32542
cerebral peduncles	31433

Tissue	Relative Expression
precentral gyrus	30153
occipital lobe	25709
Alzheimer brain frontal lobe	25532
hippocampus	25355
cerebral cortex	24834
temporal lobe	24322
frontal lobe	23988
retina	12417
Alzheimer brain	11426
pons	9090
brain	8306
thalamus	8192
aorta	7281
lung tumor	5753
spinal cord	4939
corpus callosum	4804
breast	2937
testis	2721
artery	2557
neuroblastoma IMR32 cells	2504
cerebral meninges	2487
heart ventricle (left)	2452
dorsal root ganglia	2452
ovary tumor	2369
thrombocytes	2180
vein	2150
penis	1698
fetal kidney	1585
rectum	1574

Tissue	Relative Expression
lymph node	1489
fetal brain	1361
prostate	1323
liver liver cirrhosis	1226
bone marrow CD15 ⁺ cells	1097
esophagus	1003
ileum	942
adrenal gland	849
heart atrium (left)	781
neuroblastoma SH5Y cells	724
aorta sclerotic	704
HEK 293 cells	644
small intestine	553
erythrocytes	530
breast tumor	495
lung COPD	443
cord blood CD71 ⁺ cells	410
stomach	388
colon tumor	380
heart atrium (right)	322
fetal lung	288
HEP G2 cells	280
skeletal muscle	278
bone marrow CD71 ⁺ cells	263
bone marrow CD34 ⁺ cells	261
neuroblastoma SK-N-MC cells	256
HUVEC cells	218
bladder	186
coronary artery sclerotic	185

Tissue	Relative Expression
coronary artery smooth muscle primary cells	184
fetal aorta	168
skin	165
fetal heart	142
colon	132
prostate BPH	115
pericardium	113
substantia nigra	108
lung	104
cervix	89
MDA MB 231 cells (breast tumor)	84
Jurkat (T-cells)	83
placenta	73
interventricular septum	72
leukocytes (peripheral blood)	68
stomach tumor	56
thyroid	55
ileum chronic inflammation	33
pancreas liver cirrhosis	30
uterus	29
trachea	23
liver tumor	23
adipose	21
thyroid tumor	12
heart	10
kidney tumor	9
spleen liver cirrhosis	9
salivary gland	8
coronary Artery	5

Tissue	Relative Expression
bone marrow	4
esophagus tumor	4
kidney	4
ileum tumor	3
liver	3
spleen	3
uterus tumor	3
mammary gland	2
fetal lung fibroblast cells	2
fetal liver	1
pancreas	0
thymus	0
HeLa cells (cervix tumor)	0

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CLAIMS

1. An isolated polynucleotide being selected from the group consisting of:

5 a) a polynucleotide encoding a cation transport ATPase-like protein polypeptide comprising an amino acid sequence selected from the group consisting of:

10 i. amino acid sequences which are at least about 95% identical to the amino acid sequence shown in SEQ ID NO: 2; and

15 ii. the amino acid sequence shown in SEQ ID NO: 2.

20 b) a polynucleotide comprising the sequence of SEQ ID NO: 1 or 3;

25 c) a polynucleotide which hybridizes under stringent conditions to a polynucleotide specified in (a) and (b) and encodes a cation transport ATPase-like protein polypeptide;

30 d) a polynucleotide the sequence of which deviates from the polynucleotide sequences specified in (a) to (c) due to the degeneration of the genetic code and encodes a cation transport ATPase-like protein polypeptide; and

35 e) a polynucleotide which represents a fragment, derivative or allelic variation of a polynucleotide sequence specified in (a) to (d) and encodes a cation transport ATPase-like protein polypeptide.

2. An expression vector containing any polynucleotide of claim 1.

30 3. A host cell containing the expression vector of claim 2.

4. A substantially purified cation transport ATPase-like protein polypeptide encoded by a polynucleotide of claim 1.

5. A method for producing a cation transport ATPase-like protein polypeptide, wherein the method comprises the following steps:

a) culturing the host cell of claim 3 under conditions suitable for the expression of the cation transport ATPase-like protein polypeptide; and

b) recovering the cation transport ATPase-like protein polypeptide from the host cell culture.

6. A method for detection of a polynucleotide encoding a cation transport ATPase-like protein polypeptide in a biological sample comprising the following steps:

a) hybridizing any polynucleotide of claim 1 to a nucleic acid material of a biological sample, thereby forming a hybridization complex; and

b) detecting said hybridization complex.

7. The method of claim 6, wherein before hybridization, the nucleic acid material of the biological sample is amplified.

8. A method for the detection of a polynucleotide of claim 1 or a cation transport ATPase-like protein polypeptide of claim 4 comprising the steps of:

a) contacting a biological sample with a reagent which specifically interacts with the polynucleotide or the cation transport ATPase-like protein polypeptide and

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b) detecting the interaction.

9. A diagnostic kit for conducting the method of any one of claims 6 to 8.

10. A method of screening for agents which decrease the activity of a cation transport ATPase-like protein, comprising the steps of:

a) contacting a test compound with any cation transport ATPase-like protein polypeptide encoded by any polynucleotide of claim 1;

b) detecting binding of the test compound to the cation transport ATPase-like protein polypeptide, wherein a test compound which binds to the polypeptide is identified as a potential therapeutic agent for decreasing the activity of a cation transport ATPase-like protein.

11. A method of screening for agents which regulate the activity of a cation transport ATPase-like protein, comprising the steps of:

a) contacting a test compound with a cation transport ATPase-like protein polypeptide encoded by any polynucleotide of claim 1; and

b) detecting a cation transport ATPase-like protein activity of the polypeptide, wherein a test compound which increases the cation transport ATPase-like protein activity is identified as a potential therapeutic agent for increasing the activity of the cation transport ATPase-like protein, and wherein a test compound which decreases the cation transport ATPase-like protein activity of the polypeptide is identified as a potential therapeutic agent for decreasing the activity of the cation transport ATPase-like protein.

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12. A method of screening for agents which decrease the activity of a cation transport ATPase-like protein, comprising the step of:
 - a) contacting a test compound with any polynucleotide of claim 1; and
 - b) detecting binding of the test compound to the polynucleotide, wherein a test compound which binds to the polynucleotide is identified as a potential therapeutic agent for decreasing the activity of cation transport ATPase-like protein.
13. A method of reducing the activity of cation transport ATPase-like protein, comprising the step of contacting a cell with a reagent which specifically binds to any polynucleotide of claim 1 or any cation transport ATPase-like protein polypeptide of claim 4, whereby the activity of cation transport ATPase-like protein is reduced.
14. A reagent that modulates the activity of a cation transport ATPase-like protein polypeptide or a polynucleotide wherein said reagent is identified by the method of any of the claim 10 to 12.
15. A pharmaceutical composition, comprising the expression vector of claim 2 or the reagent of claim 14 and a pharmaceutically acceptable carrier.
16. Use of the expression vector of claim 2 or the reagent of claim 14 in the preparation of a medicament for modulating the activity of a cation transport ATPase-like protein in a disease.
17. Use of claim 16 wherein the disease is a CNS disorder, a cardiovascular disorder, cancer, a genitourinary disorder or a hematological disorder.

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SEQUENCE LISTING

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 51368 Leverkusen

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- 7 -

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/01669

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/435

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, MEDLINE, SEQUENCE SEARCH, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUN XIAO LI ET AL: "Changes in levels of normal ML-1 gene transcripts associated with the conversion of human nontumorigenic to tumorigenic phenotypes." GENE EXPRESSION, vol. 8, no. 2, 1999, pages 129-139, XP009013152 ISSN: 1052-2166 the whole-document — -/-	1-12

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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- *E* earlier document but published on or after the International filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the International filing date but later than the priority date claimed

- *T* later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the International search

9 July 2003

Date of mailing of the International search report

21/07/2003

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Fax (+31-70) 340-3016

Authorized officer

Young, C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/01669

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>HALLECK MARGARET S ET AL: "Differential expression of putative transbilayer amphipath transporters."</p> <p>MOLECULAR BIOLOGY OF THE CELL, vol. 10, no. SUPPL., November 1999 (1999-11), page 79a</p> <p>XP009013156</p> <p>39th Annual Meeting of the American Society for Cell Biology; Washington, D.C., USA; December 11-15, 1999</p> <p>ISSN: 1059-1524</p> <p>the whole document</p>	1-12
X	<p>DATABASE SWISSPROT 'Online!'</p> <p>ATPase A8a2 (human), 16 October 2001 (2001-10-16)</p> <p>WALL: "ATPase class 1 type 8 member 2"</p> <p>retrieved from EMBL</p> <p>Database accession no. Q9nti2</p> <p>XP002246011</p> <p>abstract</p>	1-12
X,P	<p>EP 1 225 182 A (MILLENNIUM PHARM INC)</p> <p>24 July 2002 (2002-07-24)</p> <p>page 1 -page 5</p>	1-12


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Imprint: Bethesda, MD : American Society for Cell Biology, c1992-

URL: <http://www.molbiolcell.org/> Double click here to see full text from 1997 to present.

Notes: Title from cover.

Official publication: American Society for Cell Biology.

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Vol. 10 Suppl. (Nov 1999)

Vol. 11 Suppl. (Dec 2000)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 03/01669

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: 13-17 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 03/01669

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1225182 A	24-07-2002	1225182 A2 2002119523 A1	24-07-2002 29-08-2002

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 13-17

Present claims 13-17 relate to a product/use/method/composition defined by reference to a desirable characteristic or property, namely

the ability of a substance to reduce ATPase activity of the sequence claim 1.

The claims cover all products/compounds/methods having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product/compound/method/apparatus by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.